Rotavirus transmission in the context of reduced vaccine effectiveness in low income countries

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor of Philosophy

By

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List of abbreviations

A&E	Accident and Emergency
ADIP	Accelerated Development and Introduction Plan
AGE	Acute gastroenteritis
AIC	Akaike Information Criterion
ART	Anti-retroviral treatment
CDC	Centers for Communicable Disease Control and Prevention
cDNA	Complimentary DNA
CI	Confidence Interval
CMC	Christian Medical College
COMREC	College of Medicine Research Ethics Committee
CRF	Case record form
CSF	Cerebrospinal fluid
Ct	Cycle threshold
DAC	Development Assistant Committee
DHS	Demographic and health survey
DNA	Deoxyribonucleic acid
DRC	Democratic Republic of Congo
EDTA	Ethylenediamine tetraacetic acid
EIA	Enzyme Immune-assay
EM	Electron microscopy
EPI	Expanded programme on Immunisation
EU	European Union
GAVI	Global alliance for vaccines and immunisation
GDP	Gross Domestic Product
GPS	Global Positioning Software
HAZ	Height for age Z score
HH	Household
HIC	High income country
HIV	Human Immunodeficiency virus
IC	Internal control (for PCR extraction)
ICC	Intraclass correlation coefficient
ICT	Immunochromatographic rapid test
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IQR	Interquartile range
IU	International Unit
IV	Intravenous
Kgs	Kilograms
Km	Kilometre
LA	Latex agglutination
LIC	Low income country
Lims	Laboratory information management system
LLR	Lanzhou lamb rotavirus vaccine
LMIC	Lower middle-income country
MDG	Millennium Development Goal

Ml	Mililitre
MLW	Malawi-Liverpool-Wellcome Trust Clinical Research Programme
MRDT	Malaria Rapid Diagnostic Tests
MUAC	Mid upper arm circumference
NIH	National Institutes of Health
NPV	Negative predictive value
NSP	Rotavirus non-structural protein
NT	Non-typeable rotavirus strain
ODA	Official development assistance
OPV	Oral polio vaccine
OR	Odds ratio
ORS	Oral rehydration solution
PAGE	Polyacrylamide gels electrophoresis
PATH	Programme for Appropriate Technology in Health
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PPP	Purchasing power parity
PPV	Positive predictive value
QC	Quality control
QECH	Queen Elizabeth Central Hospital
qRT-PCR	Real-time Semi-quantitative reverse transcription PCR
R0	Basic reproductive number
RNA	Ribonucleic acid
RR	Relative risk
RRRI study	RotaRITE: Response to immunisation study
RRTE study	RotaRITE: Transmission Epidemiology study
RV1	Rotarix (monovalent) rotavirus vaccine
RV5	RotaTeq (pentavalent) rotavirus vaccine
RVGE	Rotavirus positive gastroenteritis
RVP	Rotavirus vaccine programme
SAGE	Strategic advisory group of experts
SAM	Severe acute malnutrition
SAR	Secondary attack rate
SD	Standard deviation
SI	Serial interval
SOP	Standard operating procedure
TB	Tuberculosis
UK	United Kingdom
UMIC	Upper middle-income country
UNDP	United Nations Development Programme
USA	United States of America
USD	United states dollar
VacSurv	Diarrhoeal surveillance platform at QECH
VE	Vaccine effectiveness
VIP	Ventilated improved pit latrine
VP	Rotavirus structural protein

WAZ	Weight for age Z score
WHO	World Health Organisation
WHZ	Weight for height Z Score
WT	Wellcome trust

My Role

I, Aisleen Bennett, confirm that the work conducted in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

I conducted the literature review in Chapter 1. Chapter 3 utilised pre-existing data sets from Vellore and Karonga. I performed the anti-rotavirus IgA assay for the Karonga samples as part of my MRes. I designed and performed the analysis presented in Chapter 3 with input from Ben Lopman and Nico Nagelkerke. The function for the mixture models was written by Peter Teunis. Nico Nagelkerke wrote the code for boot-strapping.

Chapter 4 utilised data from the diarrhoeal surveillance platform at Queen Elizabeth Central Hospital. This has been established for several years, and from January 2015 to March 2017 was jointly managed by Louisa Pollock and I. I redesigned and implemented data management systems, and wrote do-files for data cleaning. Data cleaning was performed by myself, Louisa Pollock and Richard Wachepa (VacSurv data manager). Louisa Pollock and I were jointly responsible for supervision and training of staff. I combined data collected from 2015 onwards with the pre-existing data and designed and conducted the analysis, adapting do-files written by Naor Bar-Zeev for analysis of earlier surveillance data.

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Chapter 8 uses data collected by the Horizontal Transmission arm of the RRTE study. I designed the study and wrote the protocols and data collection tools with input from

Louisa Pollock, Naor Bar-Zeev and Nigel Cunliffe. Demographic data and data on vaccine virus shedding in the index child were collected by the RRRI study. Data management and cleaning was as for the RRTE study. I analysed the data with support from Naor Bar-Zeev.

Laboratory analysis of samples for the RRTE study was conducted by study technicians under the supervision of myself and Louisa Pollock. Miren Iturriza-Gomara drafted the initial SOPs for analysis of stool samples and conducted primary training of the technicians. I was responsible for laboratory data analysis and quality control for the RRTE study with support from Miren Iturriza-Gomara and Khuzwayo Jere. Laboratory analysis for vaccinated infants in the Horizontal Transmission study was conducted by the RRRI study. Laboratory data management systems were designed by Louisa Pollock and I with support from Miren Iturriza-Gomara and the MLW data office.

Publications, presentations and awards arising from this work

Published manuscripts

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Manuscripts under review

Bennett A, Pollock L, Jere K, et al. Direct and indirect effects of rotavirus vaccination on rotavirus hospitalisations among children in Malawi four years after programmatic introduction. Under review, *Vaccine*

Bennett A, Nagelkerke N, Heinsbroek E, et al. Estimating the incidence of rotavirus infection in children from India and Malawi using serial anti-rotavirus IgA titres. Under review, *PlosONE*

Oral Presentations

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Abstract

Introduction. Rotavirus vaccine has been introduced into over 80 countries with substantial impact on rotavirus disease. However vaccine effectiveness is reduced in low-income countries. Patterns of rotavirus transmission could explain some of the observed reduced vaccine effectiveness, and vaccine-mediated reductions in rotavirus transmission may increase overall vaccine impact. A detailed understanding of rotavirus transmission in low income countries (LIC) is required to inform policy decisions to improve vaccine performance, however such data are currently lacking.

Methods. Mixture models were used as a novel method to estimate population level incidence of rotavirus in young children from serology data and describe transmission patterns in India and Malawi. Surveillance data from Queen Elizabeth Central Hospital, Malawi, were used to describe the ongoing burden of rotavirus disease after vaccine introduction and investigate for vaccine indirect effects. To investigate whether rotavirus vaccine could reduce the infectiousness of a child with rotavirus disease a household transmission study was conducted in Blantyre, Malawi to describe rates of rotavirus transmission from a symptomatic index child to household contacts, investigate predictors of viral shedding density in the index child and identify risk factors for transmission. In a final study transmission of vaccine virus from vaccinated infants to unvaccinated contacts was evaluated to investigate for horizontal transmission of vaccine virus.

Results. Mixture models described clear differences in patterns of rotavirus incidence in young children from India and Malawi. Analysis of surveillance data showed that rotavirus remains an important cause of hospitalised diarrhoeal disease in Blantyre despite high vaccine coverage, and identified some evidence of an indirect effect in unvaccinated infants. Household studies found a high rate of transmission of infection to household contacts (434/665, 65%) but a lower rate of transmission for disease (37/698, 5.3%). Disease severity in the index child was associated with an increased risk of transmission to household contacts, independent of viral shedding density. Rates of transmission of vaccine virus to household contacts were very low (2/151, 1.3%).

Conclusions. These studies demonstrate that rotavirus remains a significant cause of admitted diarrhoeal disease in Blantyre, Malawi and describe some evidence of a vaccine indirect effect. Transmission rates of rotavirus infection to household contacts are

associated with disease severity in the index child. As vaccine provides incremental protection against severe disease, vaccination therefore has potential to reduce the infectiousness of a vaccinated index child. Horizontal transmission of vaccine virus is infrequent and unlikely to make a substantial contribution to rotavirus vaccine indirect effects in this setting. In view of high vaccine coverage future studies should consider mathematical models to make inferences on the impact of vaccine and inform ongoing vaccine strategy.

Introduction

1.1 Virology

Rotaviruses are the major causative agent of childhood diarrhoeal disease world-wide. They were first identified in humans in the early 1970s, when the virus was visualised in duodenal biopsies of children with acute gastroenteritis (AGE) using electron microscopy(1). Rotaviruses make up one genus of the family *Reoviridae.* They consist of 11 segments of double-stranded RNA surrounded by a triple-layered protein shell; an outer capsid, inner capsid and internal core. There are 6 structural proteins (VPs) which make up the virion and a further 6 non-structural proteins (NSPs). One segment of RNA codes for at least one protein. In terms of the structural proteins, the inner layer (core) surrounding the RNA segments is formed from VP1, VP2, and VP3, and the middle layer (inner capsid) is formed by VP6. There are 7 serogroups of rotavirus (A to G) defined based on antigens expressed on the surface of this inner capsid (Fig 1.1) of which group A are the most clinically significant in humans(2–4). The serotype of the virus (G or P type) is defined by the neutralisation antigens (VP7 or VP4, respectively), which together make up the outer capsid. Rotavirus genomes can re-assort in the event of co-infection within a single cell, resulting in a wide diversity of rotavirus strains. G and P types can segregate independently of each other and the typing system therefore includes both G and P types (5).

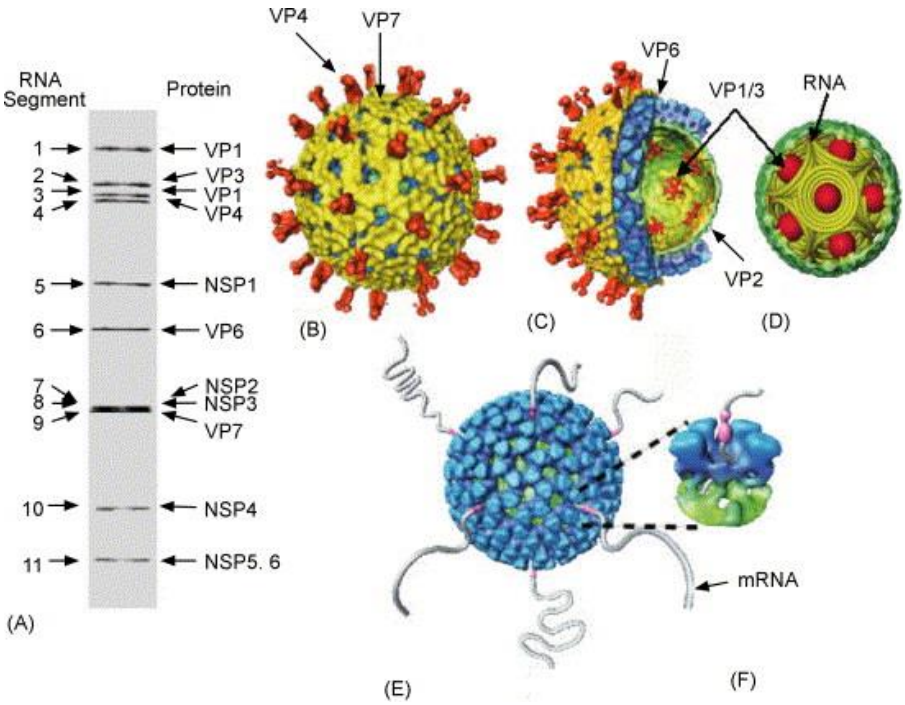

Figure 1.1 Architectural features of rotavirus. (A) PAGE gel showing 11 dsRNA segments comprising the rotavirus genome. (B) Cryo-EM reconstruction of the rotavirus triple-layered particle. VP4 is colored in orange and VP7 in yellow. (C) A cutaway view of the rotavirus TLP showing the inner VP6 (blue) and VP2 (green) layers. (D) Schematic depiction of genome organization in rotavirus. The genome segments are represented as inverted conical spirals inside the VP2 layer in green. (E and F) Model from Cryo-EM reconstruction of transcribing double layered particles (DLP), essential for rotavirus replication and assembly. *Reproduced and adapted from H Jayaram, M.K Estes, B.V.V Prasad, Emerging themes in rotavirus cell entry, genome organization, transcription and replication, Virus Research, Volume 101, Issue 1, 2004, 67–81*(6)*, with permission from Elsevier.*

1.2 Methods of rotavirus detection and characterisation

1.2.1 Electron microscopy

Electron microscopy (EM) is the historic gold standard for rotavirus detection and was the method by which rotavirus was first discovered in duodenal cells of children with gastroenteritis(1). However it requires expensive equipment and sufficient expertise to use and interpret findings, making it impractical for remote areas or for low income countries (LIC). It also cannot differentiate rotavirus groups, and is therefore unsuitable for routine rotavirus strain surveillance(7–10).

1.2.2 Antigen detection

Antigen detection methods target the VP6 protein. Examples include enzyme immunoassays (EIA), latex agglutination (LA) and immune-chromatograpic (ICT) tests. EIA typically uses 96 well plates pre-coated with an anti-rotavirus antibody to bind rotavirus

antigen. A second rotavirus-specific antibody coupled to a detector enzyme detects bound antigen, and an enzyme substrate is used to generate a colorimetric reaction(9). EIA is the currently recommended diagnostic technique for surveillance programmes due to its sensitivity, specificity, low cost and simplicity. Several commercial kits are available; the WHO currently recommends either Premier™ Rotaclone (Meridian Biosciences; Cincinnati, Ohio) or IDEIA™ Rotavirus (Oxoid (Ely) Limited Thermo Fisher Scientific, Cambridgeshire, United Kingdom)(10).

LA methods involve the reaction of rotavirus antigen with specific antibody coated onto latex particles. Agglutination can be seen visually. LA is less sensitive and specific than EIA but is faster, and does not require expensive equipment such as plate washers or spectrophotometers(11).

ICT tests are lateral flow assays, in which diluted sample migrates along a nitrocellulose membrane impregnated with gold particles via capture antibody. A control line confirms the sample has migrated a sufficient distance along the membrane and a test line contains rotavirus specific antibody which forms a complex with any rotavirus antigen present in the sample. These tests are rapid, and can be performed at point-of-care without formal laboratory facilities. ICT sensitivity compared with EIA is >90%(12–14).

1.2.3 Nucleic acid detection

Polyacrylamide gels electrophoresis (PAGE) followed by staining with silver nitrate or ethidium bromide allows direct visualisation of viral dsRNA following extraction from viral particles. Group A, B and C rotaviruses are distinguishable by their distinct migration patterns following electrophoresis. PAGE is simple enough to be used in basic laboratories, but it is reasonably time consuming. PAGE has been used in some settings for routine rotavirus detection and surveillance, differentiating Group A rotaviruses into "short" and "long" electropherotypes(10,15–17).

Molecular amplification methods (e.g. Polymerase-Chain Reaction [PCR]), where DNA is amplified following viral RNA extraction and reverse transcription (RT) (RT-PCR), are much more sensitive than antigen detection, and will detect lower viral loads, including those typically associated with asymptomatic infection. Asymptomatic infection is common in young children, and therefore these methods are not necessarily suitable for routine diagnosis of clinical disease, or for disease surveillance. In surveillance systems they are typically used for characterisation of rotavirus strains in samples which have already tested positive for rotavirus using a less sensitive technique such as EIA(5,15,18–20). Real-

time RT-PCR methods allow quantification of viral loads in clinical samples (see Chapter 2, section 2.3.4.4, page 89).

1.2.4 Rotavirus Characterisation

Because of the relevance to rotavirus vaccine design most historic surveillance programmes were primarily concerned with monitoring G types of rotavirus, however in view of the potential for separate re-assortment of G and P types and because vaccines may also target the VP4 antigen, surveillance for P types has become increasingly important(21). According to their nucleotide sequences there are at least 27 G types and 37 P types(4). Because serotypes and genotypes are equivalent for G types, G serotype types have been traditionally identified using enzyme immunoassays (EIA) with monoclonal antibodies targeting serotype specific antigens on the VP7 protein, although they can also be determined using molecular methods such as reverse-transcription polymerase chain reaction (RT-PCR). P serotypes are more challenging to predict using neutralization methods, because there are substantially more genotypes than there are reference sera determining serotype. Instead P genotypes are typically used, defined based on comparing amino acid sequences to strains with known P serotype(4)(22). Because of the challenges and investment of setting up individual assays the World Health Organisation (WHO) surveillance platform (see section 1.4.2) recommends that only one method is used for rotavirus strain characterisation, and RT-PCR genotyping is typically chosen due to its ability to determine both G and P types(10).

Non-typeable (NT) strains are not uncommonly identified when using RT-PCR methods for strain surveillance. This can arise due to variation in the VP4 and VP7 genes of common strains, such that amplification with the original primers is unsuccessful. In this case alternative primers may be required(23,24). Novel rotavirus strains can also result in failure to type. Other technical explanations include RNA degradation, low viral loads, or false positive EIA results. Sequencing can be used to identify NT strains if the presence of rotavirus RNA is confirmed. International protocols have been developed to facilitate consistent strategies of rotavirus surveillance and characterisation across countries (Section 1.4.2)(10,15,21).

1.3 Clinical features of rotavirus

Rotavirus causes a substantial spectrum of disease severity, from asymptomatic infection identified by the detection of virus in stool samples, through mild vomiting and diarrhoea which can be managed at home or the outpatient level, to severe gastroenteritis resulting

in circulatory collapse, shock, and death. Typically, rotavirus presents with fever and vomiting, then diarrhoea begins 1-2 days later. Symptoms usually resolve within 7 days. Additional clinical symptoms are usually a result of dehydration and electrolyte imbalance, and can consist of decreased urine output, lethargy, irritability, and obtundation(25).

Extra-gastrointestinal manifestations of rotavirus have been reported, most commonly seizures, which can be febrile or afebrile, and usually resolve without consequence on resolution of the rotavirus infection(26). Other reported neurological manifestation include encephalitis and cerebritis, and rotavirus has been isolated from CSF(27). Rotavirus has also been isolated from respiratory secretions(28–30), and has been associated with respiratory symptoms. Antigenaemia and viraemia is relatively common in children with rotavirus gastroenteritis(31–33) and may be associated with increased disease severity.

1.3.1 Age distribution

Almost all children will be infected with rotavirus by the age of 5 years. The peak age of clinical illness is between 4-23 months, with children from LIC typically presenting at a younger age(25,34). In preliminary data from the WHO African Rotavirus Surveillance Network over 90% of children hospitalised with rotavirus AGE were under 12 months of age(35). Neonatal infection is relatively common, but typically asymptomatic, probably due to protection arising from maternal antibodies(36). Reinfection with rotavirus is common, with disease typically reducing in severity with each subsequent episode, so that most infections in adulthood are asymptomatic(37,38) (see section 1.3.5). Asymptomatic infection also appears to decrease in frequency with increasing age; up to one third of children under two years have detectable rotavirus in their stool at any one time, compared to less than 10% in older adults(19,20). The majority of rotavirus associated death occurs in children under 5 years of age, as these are the population most at risk of significant dehydration.

1.3.2 Pathology

Rotavirus is transmitted through the faecal-oral route, requiring only a small amount of virus to cause infection(39). The stability of the triple protein coat permits transmission and passage into the proximal small intestine, where rotavirus infects the absorptive differentiated enterocytes found at the end of the villi. Rotavirus causes malabsorptive diarrhoea by a combination of mechanisms. Firstly, it destroys absorptive enterocytes,

which reduces uptake of fluid by the intestine and subsequently causes fluid loss. Secondly it leads to down-regulation of certain digestive enzymes, resulting in a higher osmotic load in the lumen contents of the small intestine and increased loss of fluid into the lumen. Thirdly, it alters the tight junctions between enterocytes resulting in fluid loss between cells. Rotavirus also causes a secretory diarrhoea via two main mechanisms; the production of an enterotoxin (NSP4) which activates chloride channels, and activation of the enteric nervous system(25,36,40,41).

1.3.3 Treatment and prevention

Once clinical rotavirus disease is established, supportive care is the mainstay of treatment. Low osmolarity Oral Rehydration Solution (ORS) should be given to all children with ongoing losses, and additional fluid given to correct dehydration and hypovolaemic shock. ORS reduces the need for intravenous (IV) fluid by up to one third, and reduces the severity and volume of vomiting and diarrhoea(42). In LIC, the degree of dehydration should be assessed and treated using WHO guidelines(43,44). These give clear criteria for the assessment of hydration status, and specific management plans depending on the presence and degree of dehydration. Children who cannot drink may require nasogastric tube rehydration and those with profuse vomiting or who are very dehydrated may require IV fluids. Where possible electrolytes should be measured and corrected, either enterally or parenterally. Oral zinc should be given to all children from LIC with acute gastroenteritis as it reduces the severity and duration of symptoms, and reduces the incidence of subsequent diarrhoea for two to three months(45). Feeding should continue, with normal feeds established as soon as the child will tolerate this. This is particularly crucial in LIC to prevent malnutrition. To the most part clinicians are not aware what the causative agent of gastroenteritis is at the time of treatment. In the absence of an aetiological diagnosis antimicrobials should only be given to children in LIC with bloody diarrhoea, with suspected cholera and severe dehydration or those with other, non-gastrointestinal, foci of infection. Large scale clinical trials examining the role of antibiotics in moderate to severe gastroenteritis disease are underway.

Strategies to reduce rotavirus disease consist of measures to reduce transmission (water, sanitation and hygiene measures), and vaccination, which will be covered in detail later in this chapter (section 1.5, page 49). It should also be noted that in LIC where access to clean water is limited, breast feeding is a crucial part of preventing diarrhoeal morbidity

and mortality. This should be exclusive (i.e. no other food or fluid) for the first 6 months of life(46).

1.3.4 Mechanisms of transmission

Human challenge studies have shown that Rotavirus is extremely infectious, with ingestion of only a few infectious particles (~10) of rotavirus sufficient to cause disease(39). Infection with rotavirus is typically via the faecal-oral route(47), but there is some evidence from a mouse model to suggest that respiratory transmission is possible(48). This has been reinforced recently by the finding that air pollution increases rotavirus force of infection(49). Rotavirus has also been detected on fomites(50,51) and on hands of care givers(51,52). It has been shown to survive for several hours on human hands, and some commonly used detergents, including soap, are inadequate in eradication of rotavirus(53–55). Outbreaks connected to water sources have been reported, and rotavirus can maintain its infectivity for several days whilst in both raw and treated fresh water(56). Furthermore, chlorine concentrations typically used for disinfecting drinking water may be inadequate against rotavirus(57).

In general, access to clean, uncontaminated water is crucial in reducing diarrhoeal disease. Adequate volumes of water are very important to maintain hygiene, as is access to appropriate toilet facilities. The extremely infectious nature of rotavirus, however, combined with its ability to survive in water and on fomites and its resistance to disinfectants means that rotavirus is not as amenable to reduction through improvements in water and sanitation as other causes of diarrhoeal disease. This is reflected by the increase in proportion of hospitalised diarrhoeal disease due to rotavirus in the last 20 years as sanitation and public health measures have generally improved(58–60), and by the fact that rotavirus remains a major problem even in countries with optimal hygiene and sanitation. Rotavirus vaccination is therefore vital in reducing transmission, preventing infection, and reducing the burden of rotavirus attributable AGE and deaths in children.

1.3.5 Acquisition of immunity to rotavirus

Several birth cohorts from different populations have monitored rotavirus infection in infants (aged under 12 months of life) and young children, and have demonstrated incremental acquisition of immunity to rotavirus disease with episodes of natural infection. Severe rotavirus disease, which is typically defined using one of two multipoint scoring systems(61), usually occurs as the first infection outside the neonatal period. In a

birth cohort in Mexico, two previous infections were sufficient to provide complete protection against subsequent severe episodes(62), however a birth cohort of 452 infants based in an urban slum in Vellore, Southern India, demonstrated that even after 3 previous infections, protection against severe disease was incomplete at 79%(38). This observation suggests that generation of robust immunity to rotavirus may require more repeated exposure in some low-income settings compared to high and middle-income settings, and is particularly relevant because rotavirus vaccine efficacy and immunogenicity is lower in low income vs high income countries (see section 1.5.3). In Vellore and Mexico anti-rotavirus IgA titres rose incrementally with age and previous infection, and demonstrated a negative relationship with infection risk, though it has not yet been possible to identify an absolute threshold in anti-rotavirus IgA titres which correlates with protection(63,64).

The mechanisms of immune protection against natural rotavirus infection are not fully understood. It is known from the cohort studies described above that natural infection provides protection against further episodes of disease, however this immunity is not sterilising, with episodes of infection continuing to occur into adulthood(19,38). Humoral immunity is thought to be most crucial to generating this protection. Serotype specific neutralising (NT) antibodies (IgA and IgG) against VP4 and VP6 have been identified following rotavirus infection, and the presence of anti-rotavirus antibodies has been shown to correlate with clinical protection against disease. Heterotypic NT antibodies have also been demonstrated, suggesting the presence of broadly reactive epitopes on the rotavirus cell surface. Rotavirus specific non-neutralizing antibodies against the RV capsid proteins VP2 and VP6 have also been identified, and these are not type specific. Anti-rotavirus IgA seems to be important in clearing rotavirus infection, however IgA deficient mice and individuals are able to eliminate rotavirus – potentially as a result of a compensatory increase in IgG(4). Rotavirus specific B cells have been identified following rotavirus infection and express gut-homing receptors ($\alpha 4\beta 7$) suggesting that they act locally in the intestine(65). In mice, the humoral response is at both the systemic and mucosal levels, B cells are necessary for long term protection against rotavirus(66), and IgA deficient mice show delayed clearance and no protection against reinfection with rotavirus(67). In terms of cellular immunity, rotavirus specific CD8+ cells are found in most adults, although rotavirus does not induce a very strong CD8 response(4). Rotavirus specific T-helper cells have been found in blood samples from children with a recent rotavirus infection and T cells are important in mice to help

remove rotavirus following an initial infection(4). The role of the cellular immune system in clinical protection against rotavirus is not yet clear. Little is known regarding the role of the innate immune system in protection against rotavirus, although upregulation of natural killer cells and increase in expression of 5 toll-like receptors (TLRs) has been observed in children following rotavirus infection(4).

1.4 Global Epidemiology of Rotavirus

1.4.1 Overview

Rotavirus is an ubiquitous pathogen causing infection and disease across the globe, however there is considerable variation in the epidemiology and impact of rotavirus in different regions of the world as a result of differences in population dynamics, economic situation, and climate. To describe this, regions are broken down by income state, using the world bank classification of low income country (LIC), Lower middle-income country (LMIC), upper middle-income country (UMIC) and high-income country (HIC). The classification of countries into these groups is reproduced in Table 1.1 The work in this thesis focuses on the epidemiology of rotavirus in a low-income, sub-Saharan African setting, but to put this in context the global epidemiology of rotavirus is outlined below. This is a narrative review. The search terms used can be seen in Table A1 (appendix, page 259). After searching, papers were manually listed, screened and reviewed by myself, and manually categorised where necessary into sub-groups. The decision to include was based on assessment of relevance and importance by myself.

1.4.2 Rotavirus surveillance systems and policy

The need to accelerate the development of effective new vaccines against rotavirus in order to reduce diarrhoeal mortality in children worldwide was highlighted in 2000, at a WHO meeting in Geneva(68). The goal of this meeting was to develop a plan of activities to expedite the development and implementation of rotavirus vaccines into LIC. Four key areas were outlined; the need for data on rotavirus disease burden and molecular epidemiology, the need for trials addressing safety, immunogenicity and efficacy of candidate vaccines specifically in LICs, strategies to address inclusion of vaccines into the WHO Expanded Programme on Immunization (EPI), and plans for issues relating to the regulation and supply requirements for vaccine introduction. Following on from this meeting the Global Alliance for Vaccines and Immunisation (GAVI) agreed to fund the Rotavirus Vaccine Programme (the RVP) and the Accelerated Development and

Introduction Plan (ADIP). The RVP was a partnership between the Programme for Appropriate Technology in Health (PATH), the Centers for Communicable Disease Control and Prevention (CDC) and the WHO, designed to facilitate data collection and communication between partner organisations and key stakeholders in industry, government and international organisations in order to support rotavirus vaccine implementation(69).

Table 1.1. Development Assist Committee list of Official Development Assistance recipients.

Least Developed Countries	Other Low-Income Countries (per capita GNI <= $1 045 in 2013)	Lower Middle-Income Countries (per capita GNI $1 046-$4 125 in 2013)	Upper Middle-Income Countries (per capita GNI $4 126-$12 745 in 2013)
Afghanistan	Democratic People's Republic of Korea	Armenia	Albania
Angola	Kenya	Bolivia	Algeria
Bangladesh	Tajikistan	Cabo Verde	Antigua and Barbuda2
Benin	Zimbabwe	Cameroon	Argentina
Bhutan		Congo	Azerbaijan
Burkina Faso		Côte d'Ivoire	Belarus
Burundi		Egypt	Belize
Cambodia		El Salvador	Bosnia and Herzegovina
Central African Republic		Georgia	Botswana
Chad		Ghana	Brazil
Comoros		Guatemala	Chile2
Democratic Republic of the Congo		Guyana	China (People's Republic of)
Djibouti		Honduras	Colombia
Equatorial Guinea1		India	Cook Islands
Eritrea		Indonesia	Costa Rica
Ethiopia		Kosovo	Cuba
Gambia		Kyrgyzstan	Dominica
Guinea		Micronesia	Dominican Republic
Guinea-Bissau		Moldova	Ecuador
Haiti		Mongolia	Fiji
Kiribati		Morocco	Former Yugoslav Republic of Macedonia
Lao People's Democratic Republic		Nicaragua	Gabon
Lesotho		Nigeria	Grenada
Liberia		Pakistan	Iran
Madagascar		Papua New Guinea	Iraq
Malawi		Paraguay	Jamaica
Mali		Philippines	Jordan
Mauritania		Samoa	Kazakhstan
Mozambique		Sri Lanka	Lebanon
Myanmar		Swaziland	Libya
Nepal		Syrian Arab Republic	Malaysia
Niger		Tokelau	Maldives
Rwanda		Ukraine	Marshall Islands
Sao Tome and Principe		Uzbekistan	Mauritius
Senegal		Viet Nam	Mexico
Sierra Leone		West Bank and Gaza Strip	Montenegro
Solomon Islands			Montserrat
Somalia			Namibia
South Sudan			Nauru
Sudan			Niue

Tanzania	Palau
Timor-Leste	Panama
Togo	Peru
Tuvalu	Saint Helena
Uganda	Saint Lucia
Vanuatu1	Saint Vincent and the Grenadines
Yemen	Serbia
Zambia	Seychelles
	South Africa
	Suriname
	Thailand
	Tonga
	Tunisia
	Turkey
	Turkmenistan
	Uruguay2
	Venezuela
	Wallis and Futuna

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(1) The United Nations General Assembly resolution 68/L.20 adopted on 4 December 2013 decided that Equatorial Guinea will graduate from
the least developed country category three and a half years after the adoption of the resolution and that Vanuatu will graduate four years after
the adoption of the resolution.

(2) Antigua and Barbuda, Chile and Uruguay exceeded the high income country threshold in 2012 and 2013. In accordance with the DAC rules
for revision of this List, all three will graduate from the List in 2017 if they remain high income countries until 2016.

In 2002 the WHO and CDC published a generic protocol for standardised surveillance for rotavirus disease in children under 5 years(70). With RVP support this was implemented across Asia, North and Latin America, parts of Europe and sub-Saharan Africa, with additional support from a regional WHO office to collate and disseminate data. In 2008 the WHO brought together existing surveillance networks to form the Global Rotavirus Sentinel Hospital Surveillance Network. This surveillance platform is used by countries from all 6 WHO regions. In 2012 169 sites from 55 countries submitted data, just under 50% of which met criteria for inclusion in analysis and reports(71).

1.4.3 Epidemiology of rotavirus in high income settings prior to vaccine introduction

In the pre-vaccine era almost all children from high income countries were infected with rotavirus by the age of 5 years. The majority of data on rotavirus incidence is derived from health care attendances and admissions, but it should be appreciated that this represents the tip of the iceberg for the enormous burden of rotavirus gastroenteritis, as illustrated

in Fig. 1.2. For community level disease and for disease requiring hospital attendance prior to the introduction of the WHO surveillance network comparisons between populations can be challenging in view of considerable heterogeneities in study design and methods used to diagnose rotavirus infection.

1.4.3.1 Frequency of rotavirus infection and disease at the community level

Prospective monitoring of an urban population in Michigan, USA from 1976 to 1981 used complement fixation to determine serological response to rotavirus and identified a 13% annual infection rate in children under 10 years of life(72). A similar study from the same population found evidence of serological infection in 21% of those under than two years of age per annum(73). In a prospective study which used EIA to examine weekly stool samples from children in day care settings over one rotavirus season in Australia, 52% of infants had at least one episode of rotavirus positivity, and 82% of these episodes were associated with symptomatic gastroenteritis(74). Similarly a study in day cares in Houston, USA, found a rate of rotavirus infection of 0.55 episodes per child year, 40% of which were associated with symptoms(75). A community study in Northern Virginia, USA, found an annual incidence of rotavirus gastroenteritis of 11/100 child years in infants, and 40/100 child years in those aged 12-23 months, where rotavirus infection was defined as rotavirus detection in stool using EM/immunoelectron microscopy and EIA, or rise in rotavirus group specific antibody in serum within 3 weeks of illness(76).

Anti-rotavirus IgA seroconversion rates from the control arms of clinical vaccine studies also give some insight into background rotavirus exposure patterns in different populations. In Europe and North America between 0 and 21% of unvaccinated infants had sero-converted following the second dose of placebo, with time post- last dose of placebo ranging from 4-12 weeks(77). It should be acknowledged however that use of anti-rotavirus IgA titres to define infection can be problematic as there is no clear evidence on how to define infection based on seroconversion, and rise in IgA titres do not necessarily correspond clearly with clinical disease(64).

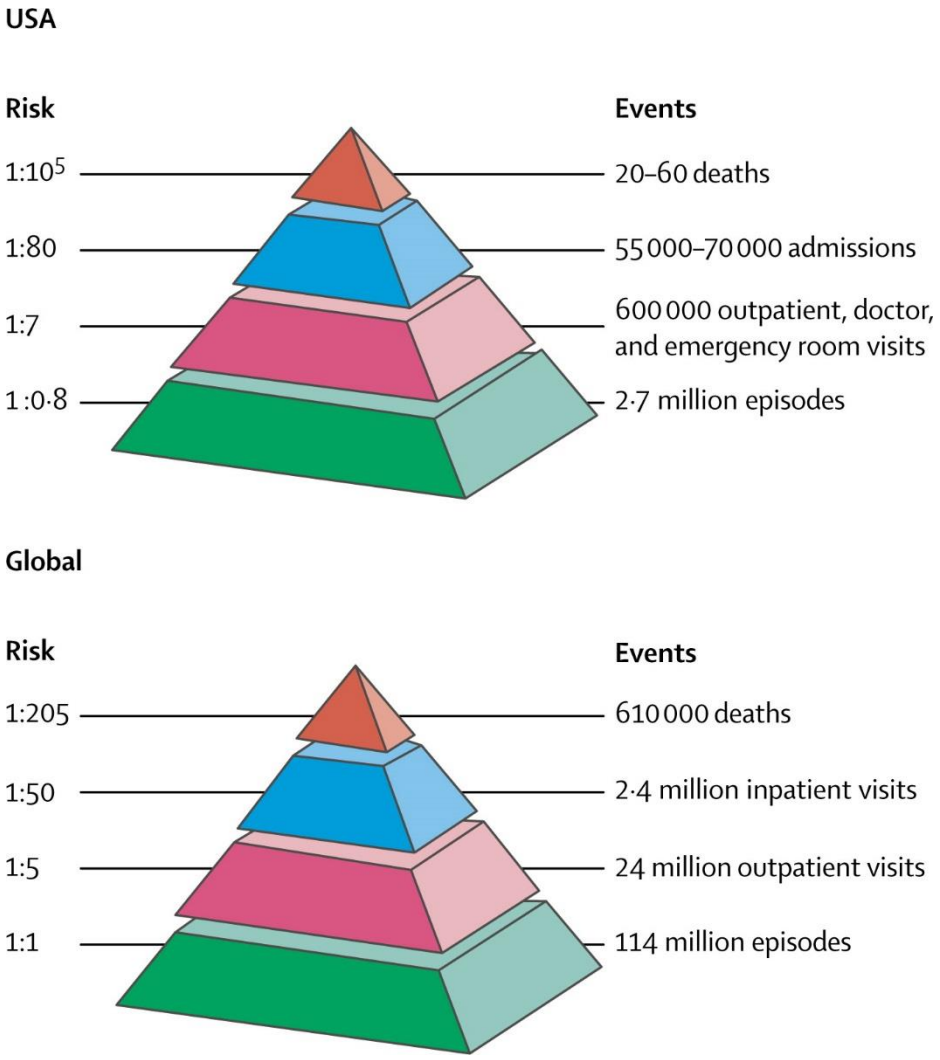


Figure 1.2 Rotavirus disease burden and risk by setting *Reproduced from Rotavirus vaccines: current prospects and future challenges. Glass et al. The Lancet* 2006 368, 323-332, with permission from Elsevier

1.4.3.2 Frequency of rotavirus disease requiring healthcare attendance

Europe

Prior to the introduction of rotavirus vaccine, rotavirus was the commonest pathogen responsible for diarrhoeal disease requiring hospital admission or outpatient clinic attendance. In 2006, the Pediatric ROTavirus European CommitTee (PROTECT study) estimated that there were 72-77,000 annual hospitalisations for community acquired rotavirus disease in those under 5 years old in the Europe Union (EU), resulting in an annual incidence of hospitalisation of 0.3-11.9/1000 (78). Williams et al in 2009 estimated the burden of rotavirus hospitalisation in the WHO European region. This region includes some countries outside of the EU, some of which are classified as low income. This study

estimated 146,287 hospital admissions, and 6500 deaths annually attributable to rotavirus, and an incidence of rotavirus hospitalisation of 1.9-4.2 per 1000 children per year, with an estimated 21.3 to 39.5% of acute gastroenteritis (AGE) admissions caused by rotavirus(79). The REVEAL study, a prospective multi-site observational study across 7 high income European countries conducted between 2004 and 2005 included outpatient attendances as well as hospitalisations, and estimated a rotavirus incidence for all health care attendances of 2.07-4.96/100 children per year(80). The REVEAL study also demonstrated a substantial impact of rotavirus disease on family life, including days off work and increased stress levels(81,82).

United States

In the United States (US) in 1996, prior to the roll out of the now withdrawn RotaShield vaccine, Glass et all reported between 54 and 186,000 annual admissions for rotavirus AGE. Between 2000 and 2006, before the introduction of the currently licensed vaccines Desai et all reported an average of 15 rotavirus hospitalisation episodes annually per 10,000 children, and estimated that vaccine introduction would lead to a reduction in hospital costs of approximately $242 million(83,84). The estimates of Glass et al were corroborated by Fischer et al, who reported approximately 60,000 rotavirus hospitalisations annually in the USA between 1993 and 2003. They also reported 37 rotavirus attributable deaths per year(85).

Australasia

In the Australian state of New South Wales, annual hospitalisation rates were estimated to be 1800 per 100,000, and Australia-wide, an estimated 10 000 children were hospitalised annually between 1998 and 2003(86,87). In New Zealand between 1998 and 2000 43% of hospitalized gastroenteritis cases were positive for rotavirus, and an estimated 634 children per 100,000 were hospitalised annually with rotavirus attributable AGE(88).

1.4.3.3 Asymptomatic infection

Asymptomatic infection with rotavirus is common in high income settings, with an age-adjusted prevalence of detectable rotavirus in stool in the United Kingdom (UK) of 11%(19) across all age ranges, and frequent asymptomatic infections detected in prospective studies in day care centres(89).

1.4.3.4 Molecular epidemiology

Prior to vaccine introduction in Europe, rotavirus genotypes G1-4 and G9 were the commonest circulating strains, with some seasonal variation and regional differences in distribution of strains between different countries in Europe – for example G1 predominated in the UK, Spain, Belgium and Sweden and G9 in Italy and France (90). EuroRotaNet, a pan- Europe surveillance system established to determine the diversity of circulating rotavirus strains in Europe included 19,140 rotavirus positive samples between 2006 and 2009 and identified substantial strain diversity, with 141 different combinations of G and P types, including both single and multiple infections. G1P[8] strains were the commonest across all three years or surveillance, followed by G4P[8], G9P[8], G2P[4], and G3P[8](91).

Similarly in the USA from 1996 to 2005, prior to vaccine introduction, G1P[8] was the commonest circulating strain at 78.5%, followed by G2P[4}, G9P[8], G3P[8], and G4P[8]. While G1P[8] was consistently the commonest detected genotype over time, prevalence of other genotypes varied(92). In Australia, G1P[8] was also the commonest strain for the majority of the pre-vaccine surveillance period, but for 2 years G9P[8] was the predominant strain and for one year G3P[8] was the dominant strain. There was also substantial year on year regional variation in the distribution of genotypes. A systematic review of global rotavirus genotype distribution from 1996 to 2007 confirmed the global predominance of G1P[8], but also noted a global declining trend in G1P[8] from the year 2000 onwards, prior to vaccine introduction(93).

1.4.4 Epidemiology of rotavirus in low income and middle income settings

1.4.4.1 Frequency of rotavirus infection and disease at the community level

Middle income settings

A birth cohort of 200 infants conducted in Mexico, an UMIC, between October 1987 and October 1988 collected weekly stool samples and 4 monthly blood samples for anti-rotavirus IgA and IgG titres for 2 years from birth. Rotavirus infection in stool was defined using EIA, and serological evidence of infection defined as a four-fold increase in IgA or IgG titres. This study found an incidence of rotavirus infection of 1 episodes per child year, and of rotavirus diarrhoea of 0.3 episodes per child year(62). 49 families of newborn infants were recruited in another UMIC, Argentina, and had serum and stool samples collected at six monthly intervals, plus stool samples collected from any household

member with diarrhoeal disease. Rotavirus was detected in stool samples using EIA, and serological infection defined as a 30% increase in anti-rotavirus IgG titres. Families were followed between May 1983 and July 1986. Incidence of rotavirus diarrhoea was 0.25 episodes per child-year for infants, and 0.04 episodes per person-year across all household members and ages. Serological evidence of rotavirus infection was estimated at 0.63 episodes per person year across all age groups(94).

A birth cohort of 452 newborns were recruited from an urban slum in Vellore, Southern India, a LMIC. Recruits were followed for 3 years between 2002 and 2006. Stool samples were collected fortnightly and serum samples every 6 months. Stool samples were screened for rotavirus antigen using EIA. Rotavirus infection in stool was defined as either rotavirus positive on two EIAs, or on RT-PCR and serological evidence of infection was defined as a 3 fold increase in anti-rotavirus IgA or 4 fold increase in IgG. Overall incidence of rotavirus infection was 0.99 episodes per child year (95% CI 0.94-1.05), and of rotavirus diarrhoea was 0.25 (95% CI 0.22-0.29) episodes per child year. This was higher in the first year of life (1.20 [95% CI 1.14-1.37] episodes of infection and 0.49 [95% CI 0.42-0.58] episodes of rotavirus diarrhoea per child year)(38).

Low income countries

A birth cohort of 200 newborns from Guinea-Bissau, a LIC, were recruited between 1996 and 1997 and followed for 2 years. Follow-up for 46 of the recruits was discontinued due to a military conflict. Stool samples were collected weekly and screened for rotavirus using EIA. No serum samples were collected. Overall incidence of rotavirus infection over the study period was 0.6 episodes per child year(95).

As in high income countries, sero-conversion rates in the control arms of vaccine trials can provide a useful insight into the prevalence of rotavirus infection in early life and allow comparison across populations. In general, sero-conversion rates in trials conducted in lower income settings in Africa and Asia were higher than those observed in high income settings, at 6-35%(77,96).

1.4.4.2 Frequency of rotavirus disease requiring hospital/clinic attendance

In low and middle income countries, the burden of rotavirus AGE is high, with higher mortality rates than seen in high income countries. In the Middle East and North Africa (Bahrain, Iran, Iraq, Israel, Jordan, Kuwait, Oman, Qatar, Saudi Arabia, Algeria, Egypt, Libya, Morocco, Tunisia and Turkey) 16-61% of all cases of AGE, and 112 deaths per

100,000 were found to be attributable to rotavirus. Mortality for this region was highest in Iraq (4723 rotavirus attributable child deaths annually)(97). Limited data are available from central Europe, which contains some high income countries such as Bulgaria, Croatia, Czech Republic, Hungary, Poland, Romania, Russia, Slovakia and Slovenia, but also some middle income countries such as the Ukraine, Georgia, Moldova, Bosnia-Herzegovina, Belarus and Albania. In a review of the data from this region from 1999 to 2009, community incidence of rotavirus diarrhoea was reported to be between 0.11-12.3 per 1000 children under 5 years annually, with the proportion of AGE cases attributable to rotavirus ranging from 22-55% per year(98).

In Latin America and the Caribbean (including Bolivia, El Salvador, Guatemala, Honduras, St Vincent, Venezuela, Chile, Nicaragua, Paraguay and Suriname) between 2005 and 2007 rotavirus was responsible for 24-47% of hospitalised AGE(99).

In Asia, the Asian Rotavirus Surveillance Network was established in the early 2000s to generate regional data on the burden of rotavirus disease in light of emerging rotavirus vaccines. This initially included China, Hong Kong, Indonesia, Malaysia, Myanmar, South Korea, Taiwan, Thailand and Vietnam. The majority of these are high or middle income countries, with the exception of Myanmar, which is a LIC. Initial data, prior to vaccine introduction, showed high levels of rotavirus in children with admitted with AGE; with an average of 45% of stool samples positive for rotavirus, ranging from 28% in Hong Kong, to 59% in Vietnam(100). A systematic review published in 2011, which included 113 studies from 2000 to 2011 from all countries in Asia, found a pooled prevalence of rotavirus in hospitalised GE samples of 37.5%, and incidence rates of hospitalisation from 2 to 20 cases per 1000 children per year(101).

Africa

A review of rotavirus disease burden in Africa between 1975 and 1992 by Cunliffe et al found between 13-55% (median 24%) of hospitalised AGE cases in children under 15 years were attributable to rotavirus and 7-40% (median 23%) of outpatient children with diarrhoeal disease. These studies used a combination of EIA, LA, EM, viral culture and Immunoelectro-osmophoresis (IEOP) to diagnose rotavirus(102). A subsequent systematic review by Sanchez-Padilla et al included studies in sub-Saharan Africa from Jan 1990 to April 2009 and performed a meta-analysis to obtain a point estimate for disease burden for different groups. Overall, almost 40% of hospitalised AGE in infants was attributable to rotavirus, dropping to 35% in children under 5 years and rotavirus

prevalence was lower in outpatient AGE compared to hospitalised AGE (33% in infants, and 22% in under 5 year olds). Rotavirus was diagnosed using EIA in the majority of studies, with a small number using LA, PAGE and EM(103).

The WHO African Regional Office Rotavirus Surveillance Network was established as part of the WHO global sentinel surveillance platform for rotavirus. Preliminary data from this network for 2006-2008, which included data from Uganda, Ghana, Kenya, Zambia, Zimbabwe, Cameroon, Ethiopia, Tanzania, Togo and Mauritius, showed findings similar to those described in the above meta-analysis, with between 29-52% of children testing positive for rotavirus(35). A review of rotavirus studies from 1976 to 2006 found a somewhat lower fraction of AGE attributable to rotavirus; between 16 (outpatient) and 32% (combined inpatient and outpatient) of children presenting with AGE were found to be rotavirus positive. In these studies Rotavirus was diagnosed using a combination of EIA, LA and EM. Despite the variation in results it is clear that prior to the introduction of rotavirus vaccine, rotavirus was a major cause of diarrhoeal disease and morbidity in Africa(104).

1.4.4.3 Asymptomatic rotavirus infection

As observed in high income settings, a proportion of reportedly asymptomatic individuals in low and middle income countries can be found to have detectable rotavirus in their stool. In Latin America, 30% of asymptomatic children from a Mexico day centre, and 21% of their adult contacts were EIA positive for rotavirus(105). In Ecuador, Lopman et al described real-time reverse transcription quantitative PCR (qRT-PCR) detectable rotavirus in 2% of household contacts of asymptomatic community control children (106). In Asia, rotavirus was detected in 13% of asymptomatic Chinese children recruited from hospital settings.

In Africa, in Burkina Faso 18% of asymptomatic community control children aged under 5 years had rotavirus in their stool on TaqMan qRT-PCR(107), and similarly approximately 20% of controls were qRT-PCR positive for rotavirus in a case control study of diarrhoeal aetiology from Tanzania(108). In a case control study from Malawi, 31% of asymptomatic control children aged under 2 years of life were rotavirus positive using qRT-PCR(20). In Zanzibar much lower levels of rotavirus (~2%) were found in asymptomatic control children(109). In a study of asymptomatic infants in Zimbabwe, 7% of children aged under 24 months were positive for rotavirus antigen in stool on EIA(110), and in 17 % of children from Nigerian day centres were EIA positive(111). Omoigberale et al described high levels

(30%) of EIA positive rotavirus infection in 821 adults and children in an urban setting in Nigeria in 1996(112).

1.4.4.4 Molecular Epidemiology

In North Africa and the Middle East G1P[8] predominated in most countries, apart from Egypt, Israel, Iraq and Kuwait, where G2P[4] was dominant. From a review of data from central Europe, in 2005/6 G1P[8] was commonest in 3 countries (Croatia, Czech Republic and Slovenia, 22-67%), but considerably less common in others (Albania and Bulgaria (7.1 and 9.1%)(98). In Latin America and the Caribbean, the pan-American surveillance network described a predominance of the globally common G1P[8], G9P[8] and G2P[4] between 2005 and 2007, with several less common strains also detected(99). A systematic review published in 2004 of studies from 1995 reporting rotavirus strain characterisation in Latin America also found a predominance of G1P[8], and G2P[4], and also G3P[8] and G4P[8](113). In Asia, a systematic review of data from 2000 to 2011 found that the commonest circulating strains were the globally common G1P[8], G2P[4], G3P[8] and G4P[8], with again significant numbers of less common strains(101).

A review of published data on rotavirus strain types circulating in Africa prior to vaccine introduction from 1997 to 2006 noted increasing diversity of rotavirus strain types. G1P[8] was the commonest G/P combination, making up 17.4% of typed samples, though this proportion was substantially lower than observed in other surveillance platforms where G1P[8] made up over 50% of strains . Also common were G2P[6] (9.6%), G8P[6] (9.4%), and G3P[8] (7%). Mixed infections, consisting of more than one G or P type were also common. Overall, circulating strains were noted to be more diverse than documented in other continents. G9, G3 and G8 were noted to have increased in prevalence in comparison to data before 2007(114). From 2007 to 2011 a high diversity of G and P combinations continued to be observed, with G1P[8] still commonest at 18.4%, followed by G9P[8] (11.7%), G2P[4] (8.6%), and G2P[6] (6.2%). G12 strains were noted to have emerged in several African countries and made up the 6th commonest G strain, at 6.2%(115). Data on rotavirus strains following programmatic vaccine introduction are only just beginning to emerge. In Malawi, following introduction of RV1 in October 2012, G1P[8] prevalence was noted to be at its lowest since any time in the historical period of surveillance (from 1997-2012), and a possible trend towards an increase in G2P[4] was noted(116).

1.4.5 Seasonality of rotavirus

Generally, seasonality of rotavirus is more pronounced in high income countries in Europe, the United States and Australasia than in LICs, where rotavirus detection is more consistent through-out the year, although endemic rotavirus circulation has also been described from several high income countries(117). Peak season for rotavirus is typically winter - November to April in the Northern Hemisphere and May to October in the Southern Hemisphere. Prior to vaccine introduction in the USA the rotavirus season would typically start in the southwest and end in the northeast of the country after an interval of some months. This has been shown to relate to annual variation in birth-rates across the country(118). A systematic review of rotavirus seasonality in tropical countries found a significant negative association between rotavirus incidence and temperature, rainfall and humidity(119). This has also been described in more temperate climates (120) (121). However a review of the global seasonality of rotavirus found that level of development was a stronger indicator of seasonality than climate or geography(117). This is corroborated by findings of a modelling study by Pitzer et al which found that the lack of seasonality in LICs could be explained by the high force of infection and high birth rates observed in such settings(122).

1.4.6 Mortality

Rotavirus remains the commonest cause of diarrhoeal mortality in children under 5 years, responsible for 37% of diarrhoea associated deaths. The most recent estimates of rotavirus mortality are for 2013 and estimated that annual global attributable rotavirus mortality in children under five years was 215,000 (range 197,000-233,000), a decline from 528,000 (range 465,000-591,000) estimated in 2000, although direct comparisons between time periods are difficult because of variation in data used and analytical methods. Consistent with previous estimates of rotavirus mortality, the vast majority of rotavirus deaths occurred in the poorest countries of the world, with most occurring in sub-Saharan Africa (an estimated 121,000 in 2013 [range 111,000-131,000], reduced from 250,000 [range 217,000-282,000] in 2000) (58,123). Rotavirus deaths in sub-Saharan Africa demonstrated a less substantial reduction than in other parts of the globe, thus the proportion of all rotavirus deaths occurring in Africa has increased from 47.3% to 56.3% in the same time period. 4 countries contribute 49% of rotavirus deaths; India, Pakistan, Nigeria and the Democratic Republic of Congo (DRC)), with India alone

responsible for 22% of all rotavirus mortality in 2013. Angola has the highest annual incidence of rotavirus deaths at 240/100,000 children under five years(58).

1.4.7 Neonatal rotavirus infection

Neonatal rotavirus infection is worthy of particular comment. Neonatal rotavirus infection has been observed world-wide(124–127), and differs clinically and epidemiologically from infection in older infants and children. Infection is usually, but not always, asymptomatic and occurs in early life, typically within the first 7 days (127). Neonatal infection occurs year round(128), and neonatal infections are often due to different genotypes of rotavirus than those circulating in the rest of the population. In neonatal nurseries a single strain will often circulate for some time(125). Most rotavirus strains infecting neonates have a P-type of P[6], although in India G9P[11] and G10P[11] have also been observed. In one study G10P[11] infections in neonates were associated with a high frequency of clinical symptoms(125). Neonatal rotavirus infections are common, with up to 44% of neonates infected in some units(125).

Neonatal infections have been of interest for several years because of their asymptomatic nature and potential to induce protective immunity and thus inform vaccine development. A cohort study conducted in Australia followed 81 babies at birth and found that 44 (54%) shed rotavirus as neonates. The cohort were followed for 3 years. Infants with a history of neonatal rotavirus infection were not protected from rotavirus infection in later life, however they did have significantly less frequent and less severe symptoms than those babies who had not been infected as neonates(124). However a study in India which followed 33 infants infected with rotavirus at birth and 300 who were not infected at birth found no difference in the frequency of rotavirus positive diarrhoea of any severity(129). A rotavirus vaccine derived from a neonatal strain (116E) was shown to have vaccine efficacy of 56%(130) and has now been introduced into the Indian vaccine schedule, and a candidate vaccine based on a neonatal strain identified in Australia is currently undergoing clinical trials(131).

1.4.8 Summary

Rotavirus is an extremely common pathogen world-wide. At the community level, up to 50% of infants and young children from high-income settings are infected with rotavirus annually. Incidence rates for community infection are even higher in low and middle income countries, with documented incidence rates for infection as high as 1.2 episodes per child-year and higher rates of sero-conversion observed in the control arm of vaccine

trials. Interpreting these observations should take into account heterogeneities in study design and uncertainty in interpretation of serological data.

In recent years data on rotavirus hospitalisation has become more standardised with the introduction of WHO surveillance protocols facilitating comparisons between populations. Prior to vaccine introduction rotavirus was responsible for hundreds of thousands of hospital admissions per year in high income settings, with considerable associated financial and social strain on families. In low and middle income countries rotavirus was responsible for up to 60% of hospital admissions for gastroenteritis, resulting in potentially catastrophic costs for families in poverty(132,133). Other key differences in rotavirus epidemiology between high and low-income settings are the greater rotavirus strain diversity observed in lower income settings, and the less pronounced seasonality in low income compared to high income settings. The most striking, however, is the huge difference in mortality. Although rotavirus is extremely common across the world, the vast majority of the mortality burden for rotavirus is concentrated in the poorest countries in the world.

1.5 Rotavirus Vaccines

1.5.1 Overview

Research to develop rotavirus vaccines began in the mid 1970's following the discovery of rotavirus, and with increasing understanding of its contribution to the global burden of diarrhoeal disease in children. The first candidate rotavirus vaccine, a tetravalent human-rhesus re-assortment live oral vaccine (RotaShield) was licensed for use in the USA in 1998 after proving highly efficacious in clinical trials in high and middle income countries. Unfortunately following routine introduction of RotaShield it was associated with the rare but potentially life-threatening condition of intussusception and was withdrawn from the market(2).

There are currently two globally licenced live oral vaccines, a monovalent live-attenuated human rotavirus vaccine (Rotarix [RV1], GlaxoSmithKline), and a pentavalent human-bovine (WC3) assortment vaccine (Rotateq [RV5] Merck and Com, Inc.). RV1 is derived from a single G1P[8] strain of human rotavirus that was attenuated by multiple passage. RV5 consists of 5 reassortants which represent the commonest human G types (G1-4) and P type ([8])(2,134–136). In addition to these vaccines, India has developed and licensed a locally produced vaccine (ROTAVAC, Bharat Biotech International) based on a single

neonatal strain of rotavirus (G9P[11])(137), and China and Vietnam have also developed and licensed local vaccines; the Lanzhou lamb rotavirus vaccine (LLR) and Rotavin-M1, respectively. LLR is produced by Lanzhou Institute of Biological Products and Rotavin-M1 by POLYVAC. There are several additional candidate vaccines currently in development or undergoing clinical trial, none of which is yet licensed for use(134).

1.5.2 Mechanisms of vaccine medicated immunity

Initially vaccine development focussed on generating multivalent vaccines (e.g. RV5) with the principle of developing strong homotypic NT antibodies against a range of the commonest circulating strains. Following observation of immunity following natural infection - where repeated infection can generate heterotypic protection- the monovalent (RV1) vaccine was developed. Clinical trials of vaccine efficacy showed that the level of clinical protection generated by vaccines did not correlate with NT type specific antibody responses, which were considerably lower than the observed level of protection(65,96). Currently serum anti-rotavirus IgA titres are thought to provide the best correlate of protection at the population level, though a protective threshold at an individual level has not been identified(64). As with clinical disease, the mechanism of heterotypic protection not fully understood, but possibilities include generation of antibodies against common antigens (e.g. VP6), or to heterotypic epitopes, or generation of an heterotypic T cell response(96). Rotavirus specific B cells have also been shown to be a weak correlate of protection following vaccination. There is currently a paucity of data on cell mediated immune response to vaccine. Understanding correlates of protection against rotavirus remains an active research topic.

1.5.3 Measuring vaccine effects

Evaluations of vaccine effects should be explicit regarding what they are measuring. Vaccine efficacy typically refers to pre-licensure evaluations of a vaccine's ability to prevent clinical disease in clinical trial conditions. Vaccine effectiveness refers to the ability of a vaccine to protect individuals from disease once implemented into a routine vaccine schedule, outside the closely controlled confines of a clinical trial, and is typically evaluated using observational studies following programmatic vaccine implementation. Vaccine impact refers to effects of a vaccine at a population level, without necessarily requiring specific information on vaccine status of individual recruits. Measuring vaccine effectiveness involves comparing vaccinated and unvaccinated individuals within one

population, measuring vaccine impact involves comparing two populations, typically the same population before and after vaccine introduction. Vaccine impact incorporates total, indirect and overall effects of vaccination (see Chapter 4, section 4.1, page 126 for a detailed overview)(138).

1.5.4 Efficacy studies for current globally licensed vaccines

RV1 and RV5 both underwent extensive pre-licensure safety and efficacy trials in high and middle income countries of over 60,000 infants each(139,140). No increase in intussusception rates of a similar magnitude to that observed with RotaShield was seen with either candidate vaccine, and nested efficacy studies demonstrated efficacy of 85% for RV1 and 98% for RV5 against severe rotavirus gastroenteritis in the first year of life. For RV1 disease severity was defined using the 20 point Vesikari score, and for RV5 it was defined using the 24 point Clark scoring system. It should be noted that there are substantial differences in the two scoring systems, with the Clark system tending to underscore disease severity in comparison to the Vesikari score(61). Importantly the monovalent RV1 provided heterotypic protection against a broad range of circulating genotypes. RV1 was first licensed in Mexico and the Dominican Republic in 2004. RV5 was licensed for use in the United States in 2006(2). As a result of the impressive efficacy demonstrated in the above trials the WHO ratified inclusion of rotavirus vaccine into the immunisation schedule of any country where it could be expected to make a substantial public health impact, but did not initially recommend its inclusion into global immunisation schedules until further data were available from lower income settings in Africa and Asia(141).

Subsequent clinical trials for both vaccines were conducted in low, middle and high income countries in Africa, Asia and South America. Overall, rotavirus vaccine efficacy was observed to be lower in low-income settings compared to high income countries. Reasons for this difference in vaccine efficacy are not known, although there are several hypotheses under consideration. These include sub-optimal immune responses in the infant as a result of HIV, malnutrition or enteropathy, interference with vaccine virus replication by oral polio vaccine or enteric co-infection, inhibition by transplacental maternal antibody, or epidemiological phenomenon such as greater force of rotavirus infection(96,142–145). A pivotal efficacy study for RV1 conducted in South Africa (UMIC) and Malawi (LIC) demonstrated vaccine efficacy against severe disease of 77% in South Africa and 50% in Malawi. Crucially, despite this lower vaccine efficacy in Malawi the

number of episodes of severe gastroenteritis prevented by vaccine was higher than that observed in south Africa, at 3.9/100 vaccinees compared to 2.5 per 100 vaccinees, because of the extremely high burden of rotavirus attributable disease in Malawi(146).

In view of this, plus additional efficacy data from Hong Kong, Taiwan and Singapore(147,148) and effectiveness data from the USA, El Salvador and Nicaragua(149) in 2009 the immunization Strategic Advisory Group of Experts (SAGE) for the WHO recommended that rotavirus vaccine be included in all national immunisation schedules. SAGE acknowledged that vaccine efficacy estimates correlated inversely with under 5 mortality and disease incidence data(139,140,146,147,150–155), but also that in countries with higher background rates of rotavirus disease and attributable mortality the potential public health benefit of rotavirus vaccines maybe greater than in other regions, because of the potential to prevent more cases. WHO-SAGE therefore strongly recommended rotavirus vaccine introduction in those countries where diarrhoeal deaths were responsible for over 10% of under 5 mortality(156,157). They recommended that RV1 be given as two doses at 6 and 10 weeks of life alongside the first two doses of DTP to ensure maximum vaccine coverage and reduce the risk of late administration beyond the approved age window of 32 weeks. A summary of the results of rotavirus vaccine efficacy trials and their relationship to income status of the country can be seen in Table 1.2.

Table 1.2 Summary of major global trials of rotavirus vaccine

Author	Date	Vaccine	Country	Economic	Control	Number	Schedule	Efficacy*	Duration of follow up
Li	2014	RV1	China	UMIC	Placebo	3333	0,1 month	75%	12m
Kawamura	2011	RV1	Japan	HIC	Placebo	765	0, 1 month	92%	24m
Phua	2009	RV1	Hong Kong & Taiwan	HIC	Placebo	10519	0, 1-2 month	96.1%	24m
Ruiz-Palacios	2006	RV1	Latin America & Finland	HIC, UMIC, LMIC	Placebo	20169	2 doses 1-2 months apart in 6-13wk infant	84.7%	12m
Linares	2008	RV1	Latin America	UMIC & LMIC	Placebo	14286	2, 4 months	83.1%	12m
Madhi	2010	RV1	South Africa	UMIC	Placebo	973	6,10,14 weeks	81.5%	12m
						971	10,14 weeks	72.2%	12m
			Malawi	LIC	Placebo	505	6,10,14 weeks	49.7%	12m
						525	10,14 weeks	49.2%	12m
Lwata	2013	RV5	Japan	HIC	Placebo	762	3 doses before 32 weeks, first dose 6-12 weeks	100%	12m
Vesikari	2006	RV5	USA, Latin America, Europe, South East Asia	HIC/UMIC 1 x LMIC (Guatemala)	Placebo	4512	3 doses 4-10 weeks apart in 6-12 week old infants	98%***	12m
Zaman	2010	RV5	Bangladesh	LIC	Placebo	1136	6,10,14 weeks	45.7 %	12m
			Vietnam	LMIC		900	6,10,14 weeks	73.2 %	12m
Armah	2010	RV5	Ghana	LMIC	Placebo	2162	6,10,14 weeks	65.0 %	12m
			Kenya	ULIC		1221	6,10,14 weeks	83.4 %	12m
			Mali	LIC		1842	6,10,14 weeks	1.0 %	12m

*severe disease (>11 on Vesikari score) **severe disease defined using score defined by Duffy et al ***severe disease defined as 24 point severity score

1.5.5 Rotavirus vaccine introduction.

Following WHO ratification, a GAVI alliance supported programme for accelerated introduction of rotavirus vaccine into eligible countries was established, initially for GAVI eligible countries in Latin America and Europe, followed by African and Asian countries. GAVI is a public-private partnership which supports and subsidizes vaccine purchase, but also supports the logistical, strategic and technical systems essential to successful implementation of a vaccine, and which are particularly challenging in LIC. As of May 2016, 81 countries had introduced rotavirus vaccine into their national immunisation programmes, nearly half of which are LIC (Fig 1.3) In addition to this Pakistan introduced rotavirus vaccine into its schedule at the start of 2017, which promises to make a substantial impact on reducing rotavirus morbidity and mortality.

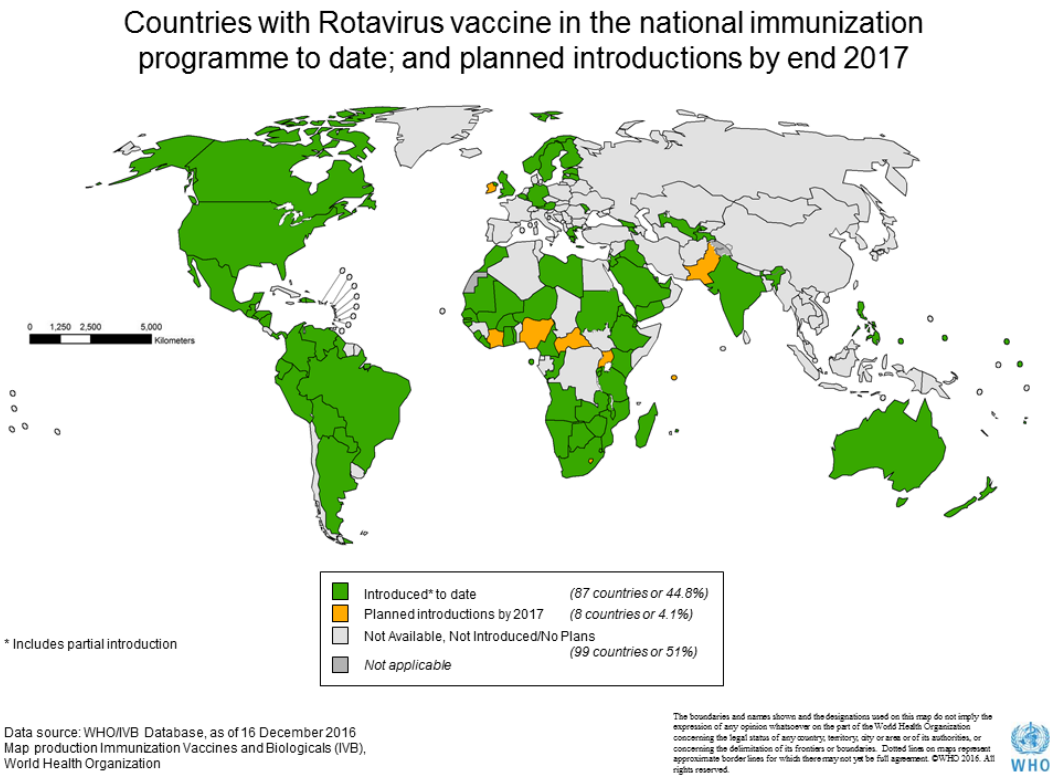


Figure. 1.3 Countries which have introduced rotavirus vaccine, reproduced from the WHO; http://www.who.int/immunization/monitoring_surveillance/en/.

1.5.6 Post implementation vaccine effectiveness and impact

1.5.6.1 High income countries

Following WHO ratification several high income countries introduced rotavirus vaccine into their immunisation schedules, with post- introduction vaccine effectiveness (VE) close to the impressive efficacy observed in clinical trials. In Europe, vaccine effectiveness and impact from 2006 to 2014 has been summarised in a systematic review by Karafillakis et al(158). Vaccine effectiveness against hospitalisation for at least one dose of vaccine in children from Austria, Belgium, Finland, Germany, Israel and Spain ranged from 89 to 96%, and for fully vaccinated children from 80% to 98%. Most studies included RV1 and RV5; one Spanish study provided separate estimates for RV1 and RV5 but found little difference between the two (RV1 98%, RV5 93% for at least one dose of vaccine). A systematic review of VE of RV5 in industrialised countries reported VE against hospitalisation of up to 100%(159).

In the USA, case-control studies of hospitalisation and emergency department visits conducted between 2006 and 2011 demonstrated vaccine effectiveness of 85-100% for RV5, 94-97% for RV1 or RV5, and 85-91% for RV1. These studies are summarised in a review by Rha et al(160). In Australia in 2007-2009 effectiveness of RV1 in indigenous children was 83-85% against severe disease, hospitalisation, or disease in under 12 month olds complicated by acidosis(161–163).

Substantial population level impact of rotavirus has also been observed following widespread introduction of rotavirus vaccine. Karafillakis et al identified and reviewed studies from Europe published between 2006 and 2014. They included 15 studies, 4 from Austria, 4 from Belgium, 3 from Finland, 1 from France, 1 from Germany and 2 from Spain. In vaccine age eligible children reductions in hospitalised rotavirus AGE ranged from 65%-84% in countries with universal vaccine introduction, and were lower but still substantial in countries with sub-national rotavirus vaccine introduction(158).

In the USA, in data from 62 paediatric hospitals, reductions in rotavirus specific hospitalisations of up to 85% were seen in 2007-2008, immediately following rotavirus introduction(164), and reductions in all diarrhoeal admissions of up to 50% noted. Desai et all estimated that for the 2008 and 2009 seasons across the US 77,000 diarrhoeal admissions were averted, equivalent to approximately $242 million in health care costs(84). A review of data from the National Respiratory and Enteric Virus Surveillance System (NREVSS) in the USA from 2000 to 2012 confirmed sustained impact of rotavirus

vaccine on the prevalence of rotavirus gastroenteritis in the 2010-2011 and 2011-2012 seasons, with a decline of up to 90% in the frequency of rotavirus positive stool samples compared to pre-vaccine data(165). Impact of vaccine introduction on the seasonality of rotavirus infection in the USA, with a shorter, blunted season, was noted soon after vaccine introduction in 2006(166), and more recently the prevalence of rotavirus has failed to meet the trigger level required to define a rotavirus season(165).

In Australia, reductions in rotavirus notifications in Queensland declined by up to 65% by 2008, following rotavirus vaccine introduction in 2007(167). Davey et al studied the rate of all-cause and rotavirus attributable non-admitted AGE presentations at Accident Emergency departments in New South Wales from 2003 to 2011 and noted a 18.3% reduction in all cause and 55.4% reduction in rotavirus presentations following RV1 introduction in 2007(168). Substantial reductions in rotavirus attributable and all-cause AGE hospitalisation rates were also observed in three Australian states (New South Wales, Australian Capital Territory and the Northern Territory) following RV1 introduction(169), and in Queensland following RV5 introduction(170).

1.5.6.2 Low and Middle income countries

Vaccine effectiveness in low and middle income countries

Comparison between studies is not straight forward as there is considerable heterogeneity in study design and analysis particularly in relation to control groups used and sub-groups used for vaccine-effectiveness estimates. Variation in sub-groups for effectiveness estimates most commonly occurred in relation to disease severity, with some studies presenting vaccine effectiveness for all rotavirus gastroenteritis, others focussing on severe disease (typically defined as a Vesikari score >= 11), and others using hospitalised gastroenteritis as an end point. There was also some variation in age groups used, with some studies describing vaccine effectiveness by age group, and others grouping all vaccine age eligible children together. Most commonly cases were vaccine age eligible children with AGE presenting or admitted to health centres. Controls usually comprised one or more of children with AGE who tested negative for rotavirus (test negative controls), asymptomatic controls from the community or children hospitalised with a condition other than AGE, for example children admitted with acute respiratory infections (ARI) Major vaccine effectiveness studies in low and middle income countries are summarised in Table 1.3 .

The first data on real world vaccine performance in low and middle income countries arose from Latin America. VE in children in the HMICs Colombia and Brazil varied considerably depending on the age group studied and the control group used. For children under one year of life VE estimates ranged from 56 to 96%(171–174) with the estimate of 56% obtained in Brazil using hospital controls, although the same population and age-group were reported to have an estimated VE of 96% using community controls. With the exception of this, all point estimates for VE from Colombia and Brazil were 78% or greater, including one study which specifically looked at the completely heterotypic G2P[4](174). All studies reported VE for RV1, and one study reported VE for RV1 and RV5 combined(172).

Findings were variable in the lower middle income countries of Nicaragua, Guatemala, Bolivia and El Salvador. VE for RV5 for infants in Nicaragua varied from 71 to 96%(149,175), but was considerably lower when all vaccine age eligible children were included (43-49%)(176). In Guatemala, Bolivia and El Salvador, VE was reported for RV1, with the exception of Guatemala which reported data on RV1 and RV5. VE in infants varied from 73-83%, and again was consistently lower (52-77%) when older age groups were included(177–180).

Recently data has been published from lower middle income countries in Europe; Armenia and Moldovia. Both of these reported data on RV1 using test negative controls, with VE estimates of 68-84% in infants, and 62-79% when all vaccine age eligible children were included(181,182).

In Africa, VE estimates appear to be lower across all income strata than those reported from Latin America and Europe. In the UMICs of Botswana and South Africa VE for RV1 ranged from 54-57%, regardless of age group(183,184). In Ghana, a LMIC, VE was 78% for infants and 60% for all vaccine age eligible children(185). The only effectiveness data available to date from LIC is from sub-Saharan Africa, with VE estimates from Malawi, Rwanda and Zambia(116,186–188). VE estimates from these countries vary from 56-75%, depending on the age category used. Malawi and Zambia report data on RV1, Rwanda on RV5. All African countries used test negative controls. Key vaccine effectiveness studies are summarised in Table 1.3

Vaccine impact in low and middle income countries

Despite the lower VE observed in low and middle income countries, programmatic rotavirus vaccine introduction has had a substantial impact on the burden of disease. In 4

countries in Latin America (Bolivia, El Salvador, Honduras, Venezuela), following introduction of rotavirus vaccine, diarrhoeal admissions in under 5 were estimated to have declined by 8%, and 12% in infants. Diarrhoeal deaths in children under 5 were also estimated to have declined by up to 58%(189). In 2008 and 2009, following vaccine introduction in 2007, rotavirus hospitalisation rates declined by 81% in El Salvador (78-84%) and diarrhoeal related hospital visits declined by 48% (47-48%)(190). In Nicaragua a decrease in watery diarrhoea in community based children under 5 years of age was observed (rate ratio 0.60, 95% CI 0.40-0.91)(191). In Mexico, all cause diarrhoeal hospitalisations declined by 40% in children under 5 years 2 years after vaccine introduction(192). At 2 years following vaccine introduction in Panama, diarrhoeal hospitalisations had declined by 37% and diarrhoeal mortality had declined by up to 54% (95% CI 48%-60%)(193,194). In Brazil, 3 years after rotavirus vaccine introduction in children under one, all cause diarrhoeal hospitalisations had reduced by 36% and mortality had halved(195). Latin America was the first region to demonstrate evidence of a substantial reduction in diarrhoeal mortality in children under 5 years following programmatic rotavirus vaccine introduction(196–199).

In LMICs in Europe population level impact of rotavirus vaccine was also observed, with a rate ratio for rotavirus admissions following vaccine introduction of 0.64(95% CI 0.56-0.74) in children under 5 years in Moldovia, and a 69% reduction in the number of rotavirus positive admissions in children under 5 years 3 years following rotavirus vaccine introduction in Armenia(181,182).

In Africa, evidence of substantial impact is beginning to emerge. In Togo, early evidence showed a decline in the proportion of children with AGE testing positive for rotavirus from 53% to 36% in the first year following vaccine introduction(200). In South Africa a 45-65% reduction in all cause diarrhoeal admissions was observed in the 4 years following RV1 introduction(201). In Rwanda, declines of almost 50% were observed in admissions for all cause AGE in the three years following rotavirus vaccine (RV5) introduction(202). In Zambia, seasonal peaks in rotavirus activity were noted to be blunted, and a decrease in diarrhoeal hospitalisation(18-29%) were observed(203). Declines in all cause and rotavirus specific hospitalisations at a population level have also been reported from Ghana(204). More recently reductions in rotavirus hospitalisations of up to 64% in children under 5 years have been observed in Zanzibar, Tanzania and declines of up to 43% have been observed in in infants presenting to Accident and Emergency departments at 3 hospitals in Zimbabwe(205,206).

1.5.6.3 Vaccine impact on mortality

Rotavirus vaccine introduction into regions with high mortality is still too recent to make substantial impacts on global rotavirus deaths, but studies from Latin America and recent data emerging sub-Saharan Africa(197,198,207,208) have shown country level declines in rotavirus attributable mortality, and as such one would expect to see this reflected in updated mortality estimates over the next ten years. Comprehensive data on impact on mortality in low income African settings are still awaited, but there is some initial data from Zambia, a LIC, and Botswana, an UMIC, to show a reduction in diarrhoeal deaths in vaccine age eligible children. In Zambia a 27-33% reduction in inpatient diarrhoeal deaths was noted, and in Botswana a 22% decline(203,207). While the global impact of rotavirus vaccine on rotavirus mortality is not yet known, modelling studies have predicted the potential for significant reductions(209).

1.5.6.4 Impact of vaccine on seasonality

Modelling studies from Pitzer et al have shown that intrinsic factors such as birth rate and transmission rates can influence seasonality, with higher birth rates and transmission rates, such as seen in low income settings, leading to a blunting of seasonal effect(117,210). If true, this could mean vaccine introduction could alter patterns of seasonality, and that more pronounced seasonality may be seen in LICs following introduction of vaccine(210). Initial data from the US following vaccine introduction demonstrated a delayed and blunted season(166). Data from LICs is not yet available due to the relatively recent introduction of vaccine, but has the potential to provide valuable data to validate models and to improve our understanding of the factors which contribute to rotavirus transmission.

1.5.7 Summary

Vaccine efficacy estimates in pre-licensure clinical trials in high-income settings demonstrated very high efficacy against severe rotavirus disease(98-100%), but vaccine efficacy estimates were substantially lower in lower income settings, with an inverse correlation with under-5 mortality rates. Despite this, because of the high burden of rotavirus attributable morbidity and mortality in such settings in 2008 the WHO recommended that rotavirus vaccine be introduced into low-income countries as a priority.

Post-implementation vaccine effectiveness studies from high-income settings have demonstrated vaccine effectiveness in line with pre-licensure efficacy estimates (85-100%), and substantial population level impact has also been described. Data from low-income countries are still emerging, but data published to date has demonstrated effectiveness estimates which typically been higher than anticipated based on efficacy studies and substantial population impact has been observed including early evidence of an impact on mortality, confirming the enormous public health potential of global rotavirus vaccination. Despite this however, effectiveness estimates remain sub-optimal compared to high-income settings and appear lowest in the poorest countries. There is also some evidence of a reduced effect in older children which does not seem apparent in high income settings. As such, there remain many important unanswered questions regarding the mechanisms of reduced vaccine effectiveness in low-income countries, the duration of protection afforded by vaccine, and true extent of population impact.

1 Table 1.3 Summary of major studies reporting RV1 or RV5 vaccine effectiveness estimates from low and middle income settings

Author	Year	Country	Vaccine	Severity	Age group	VE (%)	95% CI	Control type	Notes
Africa									
	Upper middle income								
Enane	2016	Botswana	RV1	Hospitalisation	All ≥4 months	54	23, 73	Test neg	
				Hospitalisation	4-11 months	52	8, 75	Test neg	
Groome	2014	South Africa	RV1	Hospitalisation	4-23 months	57	40, 68	Test neg	
				Hospitalisation	4-11 months	54	32, 68	Test neg	
	Lower middle income								
Armah	2016	Ghana	RV1	Hospitalisation	All VAE	60	-2, 84	Test neg	Any dose
				Hospitalisation	6-11 months	78	2, 96	Test neg	
	Low income								
Bar-Zeev	2016	Malawi	RV1	Hospitalisation	All VAE	58	20, 78	Test neg	
				Hospitalisation	<12 months	71	34, 87	Test neg	
Tate	2016	Rwanda	RV5	All	All VAE	75	31, 91	Test neg	
				All	6-11 months	65	-80, 93	Test neg	
Beres	2016	Zambia	RV1	Hospitalisation	≥6 months	56	-34,86	Test neg	
Europe									
	Lower middle income								
Sahakyan	2016	Armenia	RV1	Hospitalisation	6-23 months	62	36,77	Test neg	
				Hospitalisation	6-11 months	68	24,88	Test neg	
Gheorgita	2016	Moldovia	RV1	Hospitalisation	VAE	79	62 88	Test neg	
				Hospitalisation	6-11 months	84	67, 92	Test neg	
Latin America									
	Upper middle income								
Cotes-cantillo	2014	Colombia	RV1	Hospitalisation	VAE	-2	-182, 62	Test neg	
				Hospitalisation	6-11 months	84	23, 97	Test neg	
Ichihara	2014	Brazil	RV1 &RV5	Hospitalisation	4-23 months	74	58, 84	Hospital	
				Hospitalisation	4-11 months	78	54, 90	Hospital	
Justino	2011	Brazil	RV1	Hospitalisation	VAE≥3 months	76	58, 86	Community	
				Hospitalisation	VAE≥3 months	40	14, 58	Hospital	
				Hospitalisation	3-11months	96	68, 99	Community	

				Hospitalisation	3-11 months	56	12, 78	Hospital	
Correia	2010	Brazil	RV1	Hospitalisation	VAE≥12 months	5	-187,69	Test neg	
				Hospitalisation	VAE≥12months	41	-79,81	Hospital	G2P[4] only
				Hospitalisation	6-11 months	85	54, 95	Test neg	
				Hospitalisation	6-11 months	83	51, 94	Hospital	
	Lower middle income								
Patel	2016	**Nicaragua**	RV5	Hospitalisation	VAE	42	6, 64	Test neg	G1P[8] only
				Hospitalisation	6-11months	71	31, 88	Test neg	
Mast	2011	Nicaragua	RV5	Severe AGE	VAE	58	38, 72	Hospital	
				Severe AGE	VAE	87	78, 93	Community	
				Severe AGE	<12 months	82	60, 92	Hospital	
				Severe AGE	<12 months	96	82, 99	Community	
Patel	2009	Nicaragua	RV5	Hospitalisation	VAE	49	17, 68	Hospital	
				Hospitalisation	VAE	43	9, 64	Community	
Gastanaduy	2016	Guatemala	RV1 & RV5	Hospitalisation	VAE	52	26, 69	Test neg	
				Hospitalisation	6-11 months	73	35, 89	Test neg	
Pringle	2013	Bolivia	RV1	Hospitalisation	VAE	59	37, 73	Test neg	
				Hospitalisation	2-12 months	76	50, 89	Test neg	
Patel	2013	Bolivia	RV1	Hospitalisation	VAE	69	54, 79	Test neg	
				Hospitalisation	VAE	77	65, 84	Community	
				Hospitalisation	6-11 months	64	43, 80	Test neg	
				Hospitalisation	6-11 months	77	51, 89	Community	
De Palma	2010	El Salvador	RV1	Hospitalisation	VAE	76	64, 84	Community	
				Hospitalisation	6-11 months	83	68, 91	Community	

VE is for all doses vaccine unless otherwise specified. VAE= vaccine age eligible, controls: test neg = rotavirus negative gastroenteritis control, community= asymptomatic community control, hospital= non-gastroenteritis hospital control (e.g. acute respiratory infection)

1.6 Indirect effects of rotavirus vaccine

1.6.1 Overview

In the context of the high disease burden and reduced rotavirus vaccine effectiveness in LIC, any additional benefits of the vaccine may be particularly important to the overall population level impact and cost-effectiveness of a vaccine programme. The protective effect of a vaccine on a community can be divided into two categories; the direct effect, which describes protection afforded directly by the vaccine to the vaccinated individual, and the indirect effect of the vaccine. Vaccine indirect effects describe reduction in disease burden mediated by effects on rotavirus transmission as a result of vaccination(211); they can occur both in unvaccinated individuals, and can provide additional protection on top of direct effects in vaccinated individuals(212).

Indirect effects can be broadly divided into two categories. Firstly herd immunity, which is immunity generated in unvaccinated individuals as a result of transmission of vaccine virus within the community, and secondly herd protection, which results from a reduction in transmission of wild type infection(213). Herd protection can arise as a result of two phenomenon; a reduction in the infectiousness of a vaccinated index case if they do acquire disease, such that they infect fewer susceptible individuals than if they were unvaccinated, and/or an overall reduction in the number of infected and therefore infectious cases, with subsequent reduced likelihood that susceptible community members will come into contact with an infectious individual(214). As a general rule parenteral, killed vaccines provide herd protection only, while live, oral vaccines such as oral poliovirus vaccine (OPV) and rotavirus vaccine can provide both herd protection and herd immunity(213,215).

1.6.2 Evidence of indirect effects from HIC

Strong evidence of rotavirus vaccine indirect effects from HIC have been observed following routine use of RV1 and RV5. Although there is considerable heterogeneity among studies, and the majority of studies are based on observational, population level data, indirect effects have been observed to varying extents across a range of different high income countries, encompassing inpatient and outpatient health care settings and laboratory level data, and have been consistently detected despite a variety of different study methods.

In the USA, RV5 was recommended for inclusion in the routine national schedule in 2006, and RV1 in 2008, and studies from the USA have demonstrated clear evidence of rotavirus vaccine indirect effects across a variety of age groups using both prospective and retrospective approaches. National level health insurance data reviewed from 2002 to 2011 revealed a decline in incidence of rotavirus gastroenteritis requiring health-care attendance in non-rotavirus vaccinated Diphtheria Tetanus Pertussis (DTP) recipients from 151 per 100,000 infants pre- rotavirus vaccine introduction, to 110 per 100,000 infants post- vaccine introduction, and greater than expected according to vaccine coverage declines in laboratory detection of rotavirus was observed at national surveillance laboratories(216,217). Comparisons of the burden of rotavirus disease in unvaccinated children under 5 years of age following vaccine introduction demonstrated substantial reductions in rotavirus disease(25-77%) (218–220) and a review of the first 3 years of published data from the USA reported declines in rotavirus disease in inpatient, outpatient clinic and emergency department attendances for all children <5 years of age, and not just those eligible for vaccination(221). Payne et al investigated the presence of indirect effects by estimating an expected rate reduction based on vaccine coverage and efficacy estimates and used this to quantify indirect effects on rate of rotavirus hospitalisations in under 5 year olds; the observed decline(89%) was substantially greater than that expected (49%) (219). Notably, reductions have been observed in all age groups from young children through to older children and adults(222,223))(224).

For the most part, indirect effects in the USA have been measured by utilising data from surveillance platforms and comparing observed vaccine effectiveness to that expected based on local efficacy data, or by documenting reductions in disease burden in age groups which have not been covered by vaccination programmes. However one study from the USA compared the risk of diarrhoeal disease in households where a child had received rotavirus vaccine, to those whose child did not receive rotavirus vaccine, and found a statistically significant reduction in rotavirus specific and all-cause gastroenteritis hospitalisation rates in older siblings and parents of vaccinated infants(225). A further prospective cohort study was conducted by Panozzo et al in young children (<20 months) at the time of vaccine implementation to estimate direct VE, indirect VE, total VE and overall VE (see Chapter 4, section 4.1, page 126 for a detailed overview of these terms), using children from the pre-vaccine time period as the unvaccinated population. They estimated that in addition to direct VE, indirect effects conferred an additional 3-8%

protection to vaccinated infants to form the total VE, and estimated an indirect VE of 14-82%, depending on the year under consideration(226).

In Australia, programmatic rotavirus vaccine (RV5) was introduced into Queensland in 2007, and Lambert et al described a reduction in the proportion of stool tests positive for rotavirus in older, vaccine age ineligible children from Queensland in 2008(167). Clarke et al also reported declines in rotavirus gastroenteritis in vaccine age-ineligible children aged under 6 years in South Australia following introduction of RV5 and Buttery reported some evidence of indirect effects for rotavirus hospitalisation in children too old for vaccine from New South Wales, which introduced RV1(227,228). Importantly, taking into account indirect effects and all cause gastroenteritis over the first 6 years following vaccine introduction, Reyes et al estimated that the rotavirus vaccine programme in Australia was likely to be cost-saving(229).

Austria was the first country in Europe to implement programmatic rotavirus vaccine in 2007, and they subsequently reported a 22% decline in rotavirus gastroenteritis hospitalisations in children under 5 years who were too old to be eligible for vaccination(230). Following RV1 introduction in the UK, surveillance at a major hospital children's hospital noted an up to 70% decline in rotavirus gastroenteritis presentations in vaccine age-ineligible children aged under 5 years(231) and declines were also observed in rates of AGE presentations in children too old to be vaccinated at the primary care setting(232). Similar reductions were also observed in Belgium where both RV1 and RV5 are used(233). Overall, reductions in rotavirus disease among unvaccinated children under age 5 years of between 15 and 77%(230,232,234,235) have been observed in Europe. As in the US, indirect effects have been observed across a range of ages including older children and adults(12-16%)(232) and very young infants and neonates(236). Substantial indirect effect has been predicted by modelling studies informed by empirical data, with some suggestion that a strong initial indirect effect may be off-set some time after vaccine introduction by an increase in the burden of disease in older age groups(237–239). By way of summary, Pollard et al conducted a systematic review of vaccine effectiveness from several high and middle income countries, and estimated a median indirect effect for rotavirus attributable AGE in infants of 22%(240).

1.6.3 Evidence of indirect effect from low and middle income settings

1.6.3.1 Evidence of indirect effects from middle income settings

Evidence of rotavirus vaccine indirect effects is beginning to emerge from some UMIC and LMIC. In Latin America, in Mexico a 17% reduction in diarrhoea related hospital admissions was observed in unvaccinated children under five years following programmatic rotavirus vaccine introduction(199), and a nearly 30% decline in diarrhoeal mortality was observed in children between one and two years of age, few of whom were eligible for vaccination. In Brazil, greater than expected decreases in diarrhoeal related hospitalisations following rotavirus vaccine introduction were observed in children under five, suggesting an element of indirect protection in this age group(241).

In central Europe, there is some evidence of an indirect effect of rotavirus vaccine reported from Armenia(182), where reductions in hospitalisations of up to 30% in children too old to be vaccinated have been described, and from Moldovia where reductions in rotavirus hospitalisations following vaccine introduction exceeded expectations for all age groups under 5 years(181). In South-East Asia in data from Thailand, an up to 69% reduction in vaccine age-ineligible children aged under 5 years has been observed following vaccine introduction(242). Data from sub-Saharan Africa have, however, been less clear, with no significant reduction in rotavirus disease in unvaccinated children observed in studies from the middle income countries of Ghana or South Africa, although the data to date are limited(184,185).

1.6.3.2 Evidence of indirect effects in low income settings

Currently the only published data on indirect effects of rotavirus vaccine from LICs is observational data from Rwanda, where reductions in admissions secondary to rotavirus gastroenteritis in children under five years were observed even in those children too old to have been vaccinated(202).

1.6.4 Mechanisms of rotavirus vaccine indirect effects

The mechanisms underlying observed rotavirus indirect effects are not yet fully understood, but as explained above may include both herd protection and herd immunity. In terms of herd immunity, It is known that rotavirus vaccine virus is shed in the stool of infants to varying degrees following vaccination(243), and transmission of vaccine virus to close contacts has been documented(244), but how extensive this is and to what extent this contributes to population level protection against rotavirus is as yet unknown.

Regarding herd-protection, transmission of wild-type rotavirus seems to be strongly associated with symptomatic disease, and introduction of rotavirus infection into households seems largely dependent on the presence of a symptomatic infant (section 1.7.4)(106,245). In addition the quantity of rotavirus shed in the stool of a symptomatic child has been shown to correlate with disease severity, and rotavirus vaccine mimics natural immunity which provides incremental protection against severe disease(38,62). It follows therefore that vaccine-induced reduction in frequency or severity of clinical rotavirus disease in infants and young children may lead to a reduction of wild-type rotavirus in the community, even in the event of clinical vaccine failure. This could be particularly valuable in settings with sub-optimal vaccine performance.

1.6.5 Summary

Rotavirus vaccine indirect effects potentially have a very important role to play in the impact of programmatic vaccine introduction in low income settings where disease burden is high and vaccine effectiveness is reduced compared to that observed in higher income settings. The presence of indirect effects may determine whether a vaccine programme is cost saving, not just cost-effective. This is crucial for countries currently relying on GAVI purchase support to implement vaccine programmes(246,247). There are good observational data to support the presence of rotavirus vaccine indirect effects in high income countries, but to date there are a paucity of data from LICs. There is also a lack of data on the mechanisms which may underlie rotavirus vaccine indirect effects. There are fundamental differences between LICs and high and middle income countries in terms of disease burden, presence of co-morbidities, population structures, and living environments which mean that data from higher income settings cannot be generalised to LICs. More data on the presence and extent of indirect effects in LICs are therefore urgently needed.

1.7 Wild-type rotavirus transmission

1.7.1 Overview

Given the sub-optimal vaccine effectiveness observed in LIC, understanding rotavirus transmission is crucial. Differences in rotavirus transmission between populations may provide some explanation for the observed reduced vaccine effectiveness, and understanding drivers of transmission is key for unravelling mechanisms of rotavirus indirect effects and in evaluating the potential extent of indirect effects.

1.7.2 Important parameters in rotavirus transmission

Several key parameters describe rotavirus transmission. The secondary attack rate (SAR) describes the number of new cases derived from contact with one infectious case, and varies across populations(248). SAR allows comparison of transmission rates between populations and evaluation of risk factors for transmission. A summary of current data on rotavirus SAR can be seen in section 1.7.5. The SAR forms the basis of estimating the basic reproductive number (R0), which is defined as the number of cases derived from a single case in a *fully susceptible population*(212). R0 is important for modelling of transmission, but is almost impossible to measure directly for rotavirus because of the challenge of identifying unexposed and therefore non-immune, fully susceptible individuals. Estimates of R0 (derived from modelling studies) range from 17.6 in the UK to 191 in Malawi(210,237,249). In general, estimates of R0 are higher in low-income countries than high income countries, and higher estimates of R0 are associated with settings with higher birth rates and a higher proportion of rotavirus occurring at a young age(210,237,249).

The incubation period is defined as time from infection to onset of symptoms. For rotavirus this has been estimated to be a median of 2.0 days (95% confidence interval (CI) 1.4-2.4 days)(250). The duration of infectiousness, defined as the period of time during which an individual can transmit infection to another susceptible individual, is typically taken to be duration of symptoms (approximately 4-6 days). The serial interval (SI), defined as the number of days from the onset of symptoms in one individual to the onset of symptoms in the next individual, can therefore range from 1-9 days, and has been reported to be between 4 and 7 days in two prospective studies(76,251). In at least one study SI appeared to be longer for adults (mean 6.4 days), than children (mean 4.9 days).The rate at which susceptible individuals acquire infection is termed the force of infection(252).

1.7.3 Faecal shedding of rotavirus

Symptomatic children with rotavirus diarrhoea secrete up to $100x10^9$ virus particles per gram of stool. Pre-symptomatic shedding (before onset of diarrhoea) is common, and can precede symptoms by up to 5 days(253). Two studies from India and Australia have used molecular detection methods to investigate shedding patterns over time in children with rotavirus gastroenteritis and found that viral shedding peaks soon after symptom onset(254,255), with a rapid decline over the following 10 days. Low level shedding continues for some time after resolution of symptoms, with a median duration of

shedding of 24 days, and a range of 4-57 days in symptomatic children when viral shedding is detected using PCR(254,255). Older studies using EIA to detect viral shedding demonstrated maximum shedding rates around days 2-5 of illness, with cessation 2-3 days after symptom resolution or 7-8 days after symptom onset(256). A shorter duration of shedding was observed in asymptomatic children (median duration of shedding of 18 days, range 8-25)(255). The contribution of this extended low level shedding to transmission and propagation of infection is unclear.

It is not known if viral loads vary substantially among symptomatic children, or whether viral shedding density is associated with risk of transmission. There are also very few data on determinants of viral shedding density. However, studies from India, Malawi and the UK show that children with symptomatic disease do have substantially greater viral loads compared to those with asymptomatic infection(20,255,257), and one study from India found a significant positive association between viral shedding density and symptom severity in children with clinical disease, although this pattern was less clear in neonatal infection(258,259). There is also some evidence from Malawi that shedding post rotavirus infection detected using RT-PCR may be prolonged in children with HIV infection(260).

1.7.4 Role of infants in introducing rotavirus infection into communities

A small number of studies have attempted to investigate the route of introduction of rotavirus into closed communities. In Tecumseh from 1977-1981, Koopman et al collected paired serum samples from age stratified household members and demonstrated that older ages acquired increasing proportions of rotavirus infections within the household, with young infants seeming to acquire the majority of their infections in the community(245). Galil et al report a rotavirus outbreak in a kibbutz in 1983 which appeared to start in young children before spreading to other members of the community(261). In a study conducted between 1977-1979 Englebert et al found that the presence of an additional child under two years of life in a household was a risk factor for rotavirus infection in children presenting to an outpatient clinic with diarrhoea(262).

These studies suggest that rotavirus is mostly likely to be introduced into closed environments by infants. The only exception to this is a serological study conducted in a small community in New Zealand in 1985, where Holdaway et al proposed a model where young infants acquire infection from a reservoir in older adults in the community, and then in turn infect young adults. This hypothesis was based on identifying higher than expected IgG levels in adults over 50 years(263). Understanding which groups are

primarily responsible for driving infection is important to ensure vaccine strategy is targeting appropriate sections of the population.

1.7.5 Attack rates and risk factors for rotavirus transmission

The majority of studies of transmission are from high income settings and are household based studies. Attack rates for both rotavirus infection and disease in household contacts of children with rotavirus disease are extremely high, with child contacts typically infected more commonly than adults. A study from New Zealand recruited 47 households of children under 5 years presenting to primary health care with vomiting or diarrhoea, in 28 of which the index child was positive for rotavirus. 48% of household contacts of a rotavirus index case had stool samples which tested positive for rotavirus using EIA(251) with 75% of children showing evidence of rotavirus infection compared to 33% of adults. A significant proportion of the family contacts with detectable rotavirus had symptomatic disease (14/18 [78%] adults and 16/18 [89%] children), corresponding to disease attack rates of 67% for children and 26% for adults, respectively. The similarity in attack rates for infection and disease may at least in part reflect the fact that EIA was used to detect rotavirus as EIA typically detects rotavirus viral shedding density that correlate well with symptomatic disease(20).

In a study from the UK, Wyn-Jones et al collected stool samples and symptom information from household contacts of 5 children under 5 years admitted with gastroenteritis in the UK(264). At least one family member in all of the households was found to have detectable rotavirus in their stool, where electron microscopy was used to diagnose rotavirus. Those infected included adults and children, at least 2 of whom were symptomatic. Similar findings were observed in a family study from Denmark(265). In a prospective cohort study in Washington conducted from 1977-1980, Rodriguez et al demonstrated rotavirus in 33% of children and 12% of adults exposed to an index rotavirus case, the majority of whom developed symptoms. RV was defined using a combination of electron microscopy, immunoelectron microscopy and EIA(76). The same group also reported high attack rates from an outbreak of rotavirus in a playgroup, with 18/21 contacts (86%) developing symptoms and of 10/11(91%) of those tested having evidence of rotavirus infection on EM/ELISA or serology. These included both children in the playgroup, and adult contacts(266).

In a study from Canada published in 1979 Wenman et al prospectively followed 98 households of newborn infants for a mean follow-up period of 16.4 months, and identified

43 rotavirus infections in adults, 17 of which were symptomatic(267). Rotavirus infection in adults was associated significantly with rotavirus infection in a child in the household (36/102 [35%] of adults whose children had rotavirus infection vs 4/86 [5%] of adults whose children did not have rotavirus infection [p<0.001]). All studies from high income settings were conducted in unvaccinated populations

In comparison, there are very few studies of rotavirus transmission from middle income countries, and to our knowledge none from LICs. Lopman et al's study of household transmission of rotavirus in 40 households from Ecuador between 2011 and 2012 is the only study to have used molecular methods to detect rotavirus in the stool of household contacts, which is substantially more sensitive than non-molecular techniques to identify rotavirus. It is also the only study to be conducted after introduction of routine rotavirus vaccine into the immunisation schedule, with 85% of rotavirus positive index children vaccinated. The study identified 55% of household contacts as infected with rotavirus. They reported a statistically non-significantly higher point estimate for attack rate for infection in children under 10 years (67%) than among adults over 30 years (53%)(106). Disease attack rates were substantially lower than infection rates, and were significantly reduced in adults over 30 (9%) compared to children under 10 years (31%).

The only other study of rotavirus transmission conducted in a middle income country is from India. Banerjee et al conducted a study of household transmission of rotavirus as part of a birth cohort recruited in Vellore, Southern India between 2002 and 2003. Interestingly Banerjee et al identified markedly lower attack rates for household members of children with symptomatic and asymptomatic rotavirus infection, with only 6/560 samples positive for rotavirus, only 3 of which could be genotyped, giving a definitive attack rate of 0.54%(268). EIA was used to screen for rotavirus, before nucleic acid extraction and RT-PCR for G and P typing to confirm transmission. It is not clear why attack rates were so much lower in this population. One possibility is that they included both symptomatic and asymptomatic rotavirus infections as index cases and transmission of rotavirus seems to be related to the presence of symptoms in an index child(106). A second potential explanation is that they screened contacts using the less sensitive method of EIA before using molecular methods. This is however the only study to confirm transmission of virus using sequencing to compare the nucleotide sequences of rotavirus strains within households.

1.7.6 Known risk factors for transmission

Few studies have investigated risk factors for transmission, and only Lopman et al in Ecuador conducted multivariate analysis of predictive factors for transmission. They found that transmission was associated with symptomatic disease in the index child, with an increased risk of infection with a greater degree of symptoms(106), young age of the index child, younger age of the household contact and sharing a room with the index child(106). Other identified risk factors for transmission are crowding, both within the house and on a population level and environmental considerations such as flooding(269–271).

1.7.7 Summary of data on transmission and key questions

Following introduction of rotavirus vaccines into immunisation schedules of many low-income countries and emergence of vaccine effectiveness estimates, the focus must now shift to optimising vaccine performance and reducing the residual burden of disease. In this context understanding community level rotavirus transmission in LIC is crucial. High force of infection may be a contributing factor to observed reduced vaccine effectiveness. Reduction in rotavirus transmission is key to both the existence of any indirect effects of the vaccine and to exploring mechanisms of any such effects. An increased understanding of all of the above is necessary to fully evaluate the potential population level impact of rotavirus vaccine.

It is known that children shed large quantities of rotavirus in their stool once they have developed symptomatic gastroenteritis, and may shed for extended periods of time. What predicts viral shedding density and duration in LICs, and how density and duration relate to transmission and whether vaccine can impact on these factors and thereby interrupt transmission remains unknown. It seems likely that infants play a major role in the introduction of rotavirus infection into households in high income settings, and as such programmatic vaccination of young infants has potential to substantially reduce infection in close contacts, however data from LICs where contact patterns and social behaviour differs considerably from more developed settings are lacking.

High rates of rotavirus transmission have been described from index children to their close contacts, and child-contacts appear to be more susceptible to infection than adult-contacts. There is however considerable heterogeneity in study design and in laboratory

methods used to detect rotavirus and define infection and the majority of studies are from high income settings, with only two from middle income countries. Only one study to date has used PCR as the primary method to detect rotavirus infection in contacts of index children, and only one study has conducted multivariate analysis for risk factors for transmission. There are no data from LICs where contact patterns, potential co-morbidities and living environment differ profoundly from higher income settings, and as a result risk factors for rotavirus transmission could be very different.

1.8 Work to be presented in this thesis

This thesis aims to address some of the unanswered questions outlined above. In order to do this it is divided into two sections.

Section A:

Section A consists of two chapters both of which utilise pre-existing population level data to address broader questions around patterns of rotavirus transmission and vaccine effects. The first chapter describes patterns of force of infection in 2 different populations using a novel technique to estimate rotavirus incidence from serological data. The second uses hospital surveillance data to describe the residual burden of hospitalised rotavirus disease 4 years after programmatic rotavirus vaccine introduction in Malawi, update previously published estimates of vaccine effectiveness and to evaluate rotavirus vaccine indirect effects.

Section B:

Section B utilises primary data collected by the RotaRITE Transmission Epidemiology study (described in detail in Chapter 2) and focusses in on rotavirus transmission at a household level to investigate mechanisms of rotavirus vaccine indirect effects in a low income sub-Saharan African setting. It is made up of 4 chapters. The first 3 of these describe SAR for rotavirus infection and disease at a household level, investigate predictors of shedding density in a symptomatic index child and evaluate risk factors for rotavirus transmission from an index child to a household contact. These data are used to evaluate whether rotavirus vaccine has the potential to reduce the infectiousness of a vaccinated index child, in the event of clinical vaccine failure. The fourth chapter investigates horizontal transmission of rotavirus vaccine virus to household contacts of vaccinated infants to explore the potential for rotavirus vaccine to generate herd immunity.

The broad objectives of each of these chapters is given in the next section, and specific aims and objectives are included in each chapter.

1.9 Aims and Objectives

This thesis aims to explore rotavirus transmission in a low income country, Malawi, and to relate this to vaccine performance. These data are key to informing public health strategy to address reduced vaccine effectiveness in low income settings. Specific research questions and their corresponding objectives were:

Section A:

Research questions

1. *Can existing methods to estimate population level rotavirus incidence be improved*?
2. *What is the residual burden of rotavirus disease in Blantyre, Malawi four years after vaccine introduction?*
3. *What are the current rotavirus vaccine effectiveness estimates for hospitalised rotavirus disease in Blantyre, Malawi?*
4. *Are indirect effects of rotavirus vaccine observed in hospitalised children from Blantyre, Malawi?*

Corresponding objectives were to investigate

1. Patterns of incidence of rotavirus in different low income settings using novel methods to estimate incidence from serological data (Chapter 3)
2. Whether the prevalence of rotavirus in hospitalised gastroenteritis changes over time since vaccine introduction (Chapter 4)
3. Age stratified effectiveness estimates for rotavirus vaccine in children hospitalised with diarrhoeal disease (Chapter 4)
4. The presence of rotavirus vaccine indirect effects in hospitalised children from Blantyre, Malawi (Chapter 4)

Section B:

Research questions

1. *Could rotavirus vaccine reduce the infectiousness of a vaccinated child with rotavirus disease?*

2. *Could programmatic rotavirus vaccine introduction with monovalent rotavirus vaccine lead to herd immunity through horizontal transmission of rotavirus vaccine virus?*

Corresponding objectives were to investigate:

1. SAR for rotavirus infection and disease in household contacts of children with rotavirus disease (Chapter 5)
2. Predictors of viral shedding density in children with rotavirus disease (Chapter 6)
3. Risk factors for transmission of rotavirus infection and disease to household contacts of children with rotavirus disease (Chapter 7)
4. The proportion of household contacts exposed to a vaccinated infant who subsequently shed rotavirus vaccine virus (Chapter 8)

Chapter 2. General Methods

2.1 Overview of studies and study design

The work presented in this thesis utilises data from several different studies. These are outlined in Table 2.1

Table 2.1 Overview of studies contributing data to this thesis

Chapter	Chapter title	Study	location
3	Estimating the incidence of rotavirus infection in children from India and Malawi using serial anti-rotavirus IgA titres	Archived samples from birth cohorts from Vellore, India and Karonga, Malawi	Vellore, India Karonga, Malawi
4	Direct and indirect effects of rotavirus vaccination on rotavirus hospitalizations among children in Malawi four years after programmatic introduction	VacSurv diarrhoeal surveillance platform	Blantyre, Malawi
5	Household transmission of rotavirus in Blantyre, Malawi	RotaRITE: Transmission epidemiology	Blantyre, Malawi
6	Duration and density of rotavirus shedding in children with rotavirus disease and their household contacts	RotaRITE: Transmission epidemiology	Blantyre, Malawi
7	Risk factors for rotavirus transmission in household contacts of children with rotavirus disease in Blantyre, Malawi	RotaRITE: Transmission epidemiology	Blantyre, Malawi
8	Horizontal transmission of rotavirus vaccine virus to household contacts	RotaRITE: Horizontal Transmission study RotaRITE: Response to immunisation	Blantyre, Malawi

This chapter describes the clinical and epidemiological methods which are common to all analyses presented in the results chapters 3 to 8, and methods for the laboratory work conducted in chapters 4 to 8. Individual results chapters additionally contain detailed methods describing study procedures and statistical analyses relevant to the section.

With the exception of chapter 3, all analyses utilise data collected in Blantyre, Southern Malawi. Primary data collection for this thesis was derived primarily from the Transmission Epidemiology arm of the Rotavirus: Response to Immunisation and Transmission Epidemiology (RotaRITE) study, and is described throughout the text as the RotaRITE: Transmission Epidemiology study (RRTE). The RotaRITE study was jointly established by Dr. Louisa Pollock (WT Clinical PhD fellow) and myself to address distinct but complimentary questions relating to rotavirus vaccination in Malawi. The RotaRITE: Response to Immunisation arm formed the basis of a clinical PhD for Dr. Pollock and was designed to investigate mechanisms underpinning rotavirus vaccine failure.

Screening and recruitment processes for both arms of the RotaRITE study were integrated across all the study sites. The RotaRITE study recruited from four sites in total; Queen Elizabeth Central Hospital (QECH), Blantyre and three government Health Centres, namely Gateway, Zingwangwa and Madziabango. At the Health Centres participants were screened and recruited directly. At QECH the RotaRITE study was nested within an existing diarrhoeal surveillance platform which has been in place since 1997. Enhanced surveillance at QECH was introduced on 1st January 2012 prior to programmatic introduction of rotavirus vaccine on the 29th October 2012 with the aim of monitoring vaccine effectiveness . This formed part of a Wellcome Trust Programme Grant "New Childhood Vaccines for Malawi" [VacSurv] NHSRC #867). Children recruited by the VacSurv platform were assessed for eligibility to participate in the RotaRITE study. Dependent on vaccination status and results of rotavirus diagnostic tests, children were eligible for one or both of the study arms (Fig 2.1). This is described in detail in the methods section of chapter 5 (section 5.2, page 154).

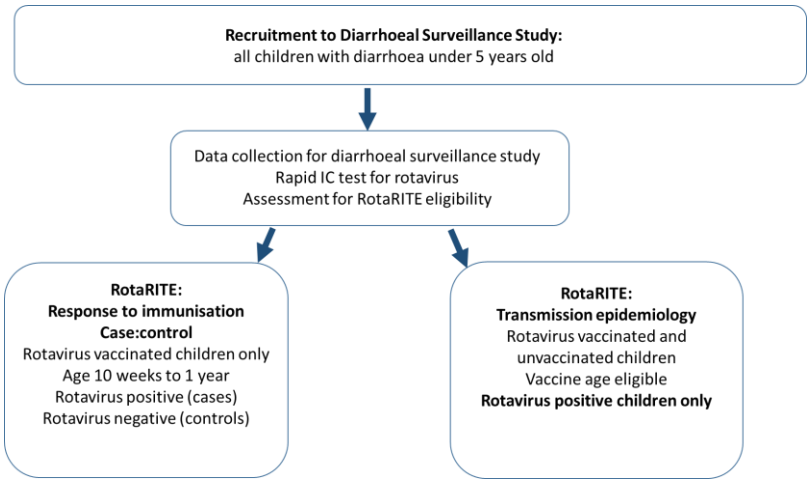


Figure. 2.1. Structure of study recruitment at QECH. Eligibility criteria for the RotaRITE Transmission epidemiology arm are outlined in chapter 5, section 5.2.7.4, page 157. IC test refers to immunochromatograpic rapid test for rotavirus.

The RotaRITE transmission epidemiology study was a cohort study that recruited eligible children presenting to any of the study sites with rotavirus positive gastroenteritis (index child). Following consent of the index child, their household contacts were recruited and followed up for 12 days after the onset of symptoms in the index child in order to determine SAR for rotavirus within households. Data were collected from household contacts on symptoms of gastroenteritis and two stool samples were collected and tested for the presence of rotavirus. A smaller nested cohort of households had more intensive

and prolonged follow up to study viral shedding in more detail. The Horizontal Transmission study was a subset of the RotaRITE Transmission Epidemiology study which recruited household members of vaccinated infants to investigate for horizontal transmission of vaccine virus.

2.2 Study site and population

2.2.1 Blantyre, Southern Malawi

Malawi is a small, land-locked country in sub-Saharan Africa with an estimated population of 16.7 million people(272). It has a sub-tropical climate, with an annual rainy season typically from November through to March. According to the 2015 Human Development report of the United Nations Development Programme (UNDP), Malawi ranks 173 out of 188 countries and territories in the Human Development Index, with 66.7% of the population defined as living in poverty and 24.5 near poverty, where poverty is defined using a multidimensional poverty index(273). The Gross Domestic Product (GDP) per capita, based on purchasing-power-parity (PPP) and international United States dollars (USD) for 2015 was 1183.6, an increase from 482.5 in 1990 (274). Under 5 mortality per 1000 live births for 2015 was 64 (90% CI 47, 91)(275), representing a dramatic reduction from 247 (234-262 90% CI) in 1990.

Notably Malawi was one of only 32% of countries to meet Millennium Development Goal (MDG) 4; to reduce by 2015 under 5 mortality rate by two thirds since 1990 levels(276). This was achieved through a combination of nutritional programmes, vaccine programmes, roll out of malaria prevention and treatment strategies and programmatic initiation of Anti-Retroviral Treatment (ART). Despite this, there are still many improvements to be made(272). Paucity of clinicians, lack of ongoing training and lack of resources mean that available medical care remains substandard compared to high income settings, and children still routinely die of preventable medical conditions such as diarrhoeal disease, pneumonia, and malaria.

Blantyre is the commercial capital of Malawi, located in the southern region (Fig. 2.2), with an estimated population of 956 898 of whom approximately 154 792 are aged under 5 years(277).

Figure 2.2 Map of Malawi. Blantyre highlighted in red.

Map data ©2017 Google

2.2.2 Malawi-Liverpool-Wellcome Trust Clinical Research Programme

The Malawi-Liverpool-Wellcome Trust Clinical Research Programme (MLW) was founded in 1995 with the aims of pursuing scientific excellence, improving the health of people in sub-Saharan Africa, and maintaining partnership between the Liverpool School of Tropical Medicine, University of Liverpool and the College of Medicine, Blantyre, Malawi. Its major funder is the Wellcome Trust, and its major research themes are preventing death from severe infections; transmission reduction in HIV, TB and Malaria; and origins and intervention in chronic disease. MLW has a strong history of collaborative research within the Malawian community and provides extensive logistical infrastructure including research laboratories and clinical research facilities. The research institute consists of local and international scientists across a multitude of disciplines and a range of seniority. MLW

is an affiliate of the University of Malawi College of Medicine as well as the University of Liverpool and Liverpool School of Tropical Medicine.

Photograph 2.1 Malawi Liverpool Wellcome Trust Clinical Research Programme

2.2.3 Queen Elizabeth Central Hospital

Queen Elizabeth Central Hospital (QECH) is the major referral hospital for Southern Malawi, with an estimated catchment area of approximately 1.3 million for Blantyre district(277). The paediatric department includes an Accident and Emergency department (A&E), neonatal unit, general paediatric wards, oncology, orthopaedics, surgery, and a nutritional rehabilitation unit. On a typical day there are between 200 and 300 inpatient children, and almost 25,000 admissions per year. Children with gastroenteritis are triaged and have treatment initiated in the A&E department. If admission is required they are usually admitted in Paediatric Special Care ward (over 6 months of age), Paediatric Nursery (previously discharged neonates to infants 6 months of age), and Moyo House (nutritional rehabilitation for children over 6 months with WHO defined Severe Acute Malnutrition (SAM)).

Children are cared for by government appointed nursing staff and patient attendants. Paediatric consultant rounds occur on a daily basis, with all critically unwell children receiving twice daily consultant review. Health care provider initiated HIV testing of both patients and their guardians at any contact with health care provision is part of routine

clinical care in Malawi, and this is undertaken at QECH by government trained HIV testers and counsellors(278). Health care is free at the point of delivery. Paediatric medical care in Malawi remains basic, with medicines limited to a few antimicrobials and anti-malarials, and very limited intensive care facilities available in the country. Drug stock-outs are frequent. Access to health care often poses a challenge with blockades at social, financial and logistical levels.

Photograph 2.2 Queen Elizabeth Central Hospital Adult Accident and Emergency Department

Photograph 2.3 Queen Elizabeth Central Hospital – Paediatric Special Care ward

2.2.4 Health Centres

In Malawi, health centres provide primary, and sometimes secondary referral level medical care. Children are brought to health centres for routine vaccinations and nutritional assessments, and for treatment when clinically unwell. Health centres are typically staffed by clinical officers who are healthcare providers with a qualification in clinical care, but who are not qualified as physicians. Children are seen and treated at these health centres on an outpatient basis; if a child requires admission he or she will usually be referred to the nearest district hospital. Antenatal and post-natal care of pregnant women typically occurs at the health centre level.

2.2.4.1 Gateway Health Centre

Gateway health centre is located adjacent to QECH (Fig 2.3). It acts as a primary health centre to reduce the burden on QECH, and as such it has no formal catchment area. It mostly provides treatment for acutely unwell adults and children. There are no antenatal or inpatient facilities. It typically sees around 6000 to 10,000 outpatients per month, and reports up to 250 cases of diarrhoea in children aged under five per month. There is a laboratory which can perform Malaria Rapid Diagnostic Tests (MRDT) and a dispensary. Recruitment to the RotaRITE study from Gateway commenced in September 2015.

2.2.4.2 Zingwangwa Health Centre

Zingwangwa Health Centre is the primary health centre for Zingwangwa district, with a catchment area of 145,821 people (Fig 2.3). It offers maternity services including antenatal and post-natal care, and runs a vaccination clinic. It also provides an HIV testing service, and has a laboratory which can perform basic tests such as Malaria Rapid Diagnostic Tests (MRDT) and a small pharmacy. The health centre has 2000 to 3500 outpatient attendances per month, reports 100-250 diarrhoeal cases per month in children under five, and vaccinates between 300-550 infants per month with rotavirus vaccine. Recruitment to the RotaRITE study commenced here in March 2015.

Photograph 2.4 Zingwangwa Health Centre

2.2.4.3 Madziabango Health Centre

Madziabango Health Centre provides primary health care to Madziabango district with a catchment area of 10,503 individuals. It is situated on the border of Blantyre district, on the main road from Blantyre to Chikwawa (Fig. 2.4). It runs antiretroviral treatment (ART) clinics for patients with HIV, general medical clinics, provides maternity care and vaccine clinics and also has a small diagnostic laboratory and pharmacy. It typically treats 2000-5000 outpatients per month. It has no electricity supply, and there was a vaccine stock out in the last six months of 2015 resulting from breakdown of a gas fridge. This included rotavirus vaccine. The RotaRITE study recruited here from August 2016.

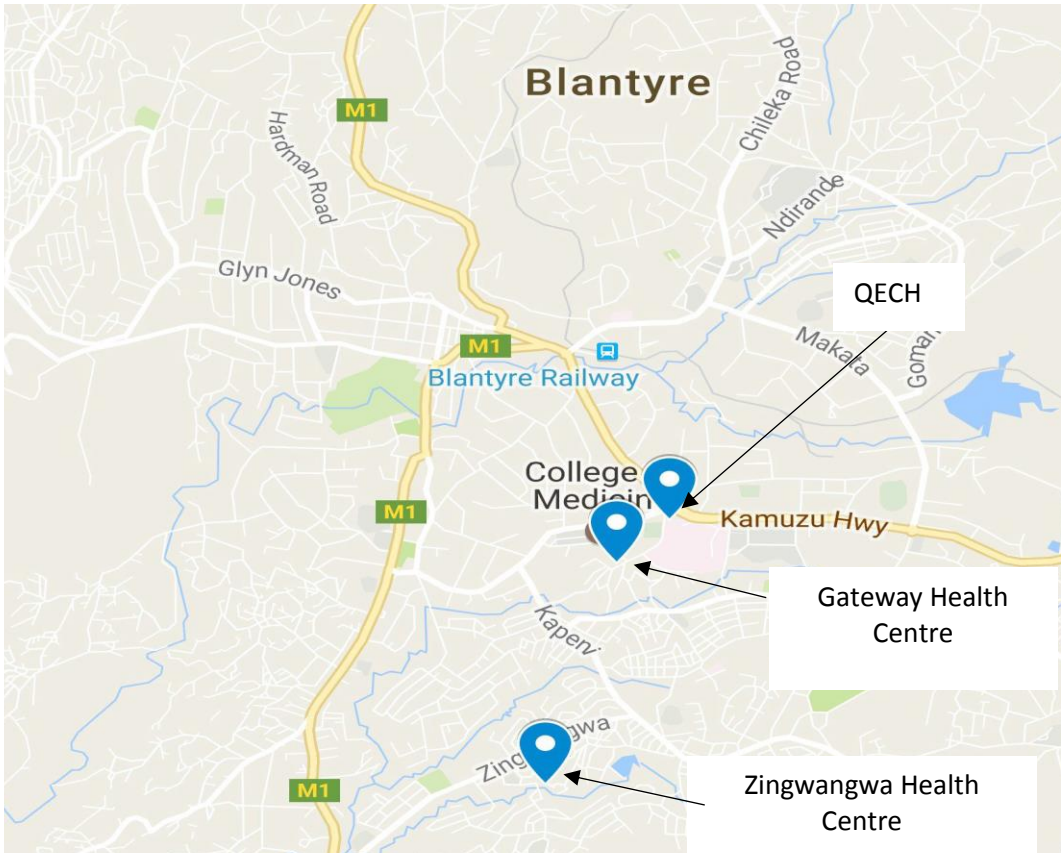


Figure 2.3. Map of recruitment sites: Gateway, Zingwangwa and QECH.

Map data ©2017 Google

Figure 2.4 Map of recruitment sites: Madziabango Map data ©2017 Google

2.2.5 Field Work

The RotaRITE: Transmission Epidemiology study involved substantial amounts of field work to recruit and follow up households. This required travel across the whole of Blantyre district using a combination of 4X4 vehicles and public transport, and often walking for long distances when houses were particularly inaccessible. Recruiting household members often required several home visits and intense follow up from the field work team.

Photograph 2.5 A study vehicle tracing a recruit

Photograph 2.6 The field team collecting assent from a recruit to the RotaRITE:TE study. *Written consent for photograph obtained from participant and guardian.

2.2.6 Study Team

The RotaRITE study team was responsible for study activities for RotaRITE Transmission Epidemiology, RotaRITE: Response to Immunisation and the RotaRITE Horizontal Transmission study. They consisted of:

Two laboratory technicians: Responsible for sample processing and storage, RNA extraction and cDNA synthesis, qRT-PCR, qualitative PCR and EIA testing of stool

Senior research nurse: Responsible for recruitment and clinical procedures required as part of the RotaRITE studies across study sites, and co-ordination of other nursing staff and activities

Five research nurses: Responsible for screening and consenting study participants and collection of data and clinical samples from children recruited into the diarrhoeal surveillance programme and

RotaRITE studies. Also responsible for bed side stool testing with immunochromatographic tests for rotavirus.

Seven field workers: Field workers were responsible for follow up and recruitment of household contacts, and collection of data and stool samples from household members.

Data manager (part time): A data manager was shared between the RotaRITE studies, diarrhoeal surveillance and a pneumonia surveillance platform. He was responsible for the flow of Case Record Forms (CRFs) for the diarrhoeal surveillance platform, scanning in of CRFs for the RotaRITE studies, and assisting with data cleaning for the diarrhoeal surveillance and RotaRITE studies.

2.3 Laboratory Methods

2.3.1 Overview of laboratory methods

All laboratory samples were processed, stored and analysed at the Malawi-Liverpool-Wellcome Trust Research laboratories. Assays were conducted by laboratory technicians line managed by myself and Dr Louisa Pollock, in conjunction with the molecular laboratory manager and core senior technicians. SOPs were written, reviewed and approved by a combination of Professor Miren Iturriza-Gomara (MIG), Dr Louisa Pollock (LP), Dr Khuzwayo Jere (KJ) and myself (AB).

2.3.2 Laboratory Processing

All samples were identified using individual bar codes attached to the sample vial. These were attached at the point of sample collection, and matching barcodes attached to corresponding data collection forms. Samples were tracked through the lab using their barcodes. On arrival into the laboratory samples were entered into the electronic "LIMS" system, which tracks samples and records selected test results.

2.3.2.1 Stool samples

On arrival stool samples were stored at 4^{o}C. A small portion of each whole stool was used to make stool suspensions. 10-20% stool suspensions were generated by adding 200ul of liquid sample (or 1 bacteriological loop if more solid) to 2ml of PBS solution and vortexing to generate a homogenous solution. The remainder of the sample was stored as whole stool. Whole stool and stool suspensions were both stored in 2ml polypropylene tubes at -80^{o}C until batch testing.

2.3.2.2 Blood samples

Blood samples were collected in 1.3ml EDTA bottles. On arrival in the laboratory blood was centrifuged at 3000rpm at 4^{0}C for 10 minutes. Following separation plasma was pipetted into 2ml screw-top

polypropylene tubes and stored at -80^{0}C until further testing. These samples are planned for testing for anti-rotavirus IgA antibodies at a later date and these data are not included in this thesis.

2.3.3 Laboratory data management

All samples were entered into the generic Laboratory Information Management System (LIMS). Sample storage plans, laboratory work flow and laboratory results were initially generated in excel spreadsheets. Approximately 12 months into the study these were transferred into an access database with a SQL server backend, specifically designed for the RotaRITE studies, and built by the MLW data department. This database now serves as a template for all new studies commencing laboratory work at MLW. Sample IDs were entered into the database using a barcode reader to read the barcode on the sample, and a work plan generated depending on the sample type. Samples were batch tested using qRT-PCR each time a box of samples was full. Input for the PCR machines was generated automatically from the work flow plan. Output from the RT-PCR machine was in the form of excel spreadsheets which were directly uploaded into the results database and linked back to the sample ID. The system was designed to facilitate flow of large volumes of data in an automated fashion and to avoid manual entry of data wherever feasible.

2.3.4 Molecular methods

2.3.4.1 Summary

RNA was extracted from stool samples, and converted to complimentary DNA (cDNA) via reverse transcription. Semi-quantitative RT-PCR (qRT-PCR) was used to detect and quantify rotavirus (Rotavirus VP6 qRT-PCR), confirm rotavirus detection (Rotavirus NSP3 qRT-PCR)), and detect Rotavirus vaccine virus (Rotavirus NSP2 qRT-PCR). Qualitative PCRs were used for strain characterisation. Details of the assays are given below.

2.3.4.2 RNA Extraction from stool suspension

Viral RNA was manually extracted from 10% stool suspension using Qiagen Viral RNA Mini-kits. Internal controls (Primer design RNA internal extraction control kit) were added to each sample for quality control purposes and tested with a separate qRT-PCR. Positive and negative controls were included in each extraction run. The positive control was a known rotavirus positive sample. The procedure was conducted in a designated clean room and surfaces were cleaned with RNAse-away spray prior to each extraction to minimise risk of contamination. Internal control (IC) RNA was prepared according to manufacturer's instructions and added to the extraction buffers. Extraction was then conducted according to protocol.

2.3.4.3 Reverse Transcription

Viral RNA was converted to complimentary DNA(cDNA) to allow amplification of RNA targets via reverse transcription. cDNA was generated using random primers (Invitrogen, ThermoFisher, Waltham, Massachusetts, USA) and reverse transcriptase enzyme (M-MLV). Random primers were used to generate cDNA which could be used for amplification of a range of targets. The cDNA generated was then archived to allow further testing at a later date if required. Reaction mix for the reverse transcription (RT) reaction was prepared as outlined in Table 2.2.

Table 2.2 Reaction mix for reverse transcription

	µl /reaction	µl /94 reactions
10X PCR buffer (without $MgCl_2$; AB)	7	658
25 mM $MgCl_2$ (AB)	14	1316
Random Primers @50 M (Invitrogen/AB)	1	94
dNTPs (10 mM; AB)	1	94
M-MLV (200U/ul)	2	188
Nuclease free Water	5	470
TOTAL	30	2820

Following preparation of the master mix the following steps were performed:

1. 40 µl of total nucleic acid was added to each well of 96 well plate and denatured at 97^{o}C for 2-5 minutes before transferring to a cool box/ice
2. 30 µl of RT master-mix was added to each well
3. Incubation step 1. – room temperature (23± 5^{o}C) for 5 minutes
4. Incubation step 2 – 37^{o}C for 60 minutes (RT isothermal reaction)
5. Inactivation step - heat at 95^{o}C for 2-5 mins

2.3.4.4 Rotavirus VP6 qRT-PCR

This semi-quantitative real time PCR (qRT-PCR) assay was used to amplify a 379 base-pair (bp) fragment of the rotavirus inner capsid protein (VP6) encoding gene from nucleic acids converted into cDNA. qRT-PCR detects amplification of target DNA as the reaction progresses, in contrast to conventional PCR which detects the product at the end of the reaction. This allows quantitation of the product under investigation, beyond a threshold level of DNA. The number of PCR cycles needed to raise copy numbers of the target sequence in a sample above a pre-defined threshold is termed the

cycle threshold (Ct), and is inversely proportional to the amount of virus present, such that lower Ct values represent a higher viral load. All qRT-PCR assays in this study were run for 40 cycles.

A standard curve (serial dilution of amplification target for which the concentration is known) was included in each run to allow estimation of the rotavirus viral load (copy numbers). Assays were conducted on the ABI 7500 PCR machine. A separate PCR was conducted for the internal extraction control (IC). Composition of primers and probes are listed in Table 2.5

Master mix was prepared and stored at -20^{o}C. Master mix for VP6 and IC reactions were prepared as listed in tables 2.3 and 2.4

Table 2.3 Reaction mix for VP6 PCR

Reagents	µl /reaction	ul /94 reactions	ul /37 reactions
Master Mix (with low Rox)	12.5	1175	462.5
VP6 F^{i} (@20 pmol/ul)	0.5	47	18.5
VP6 R (@20pmol/ul)	0.5	47	18.5
VP6 probe (FAM_MGB @ 20uM)	0.25	23.5	9.25
Nuclease free Water	8.75	822.5	323.75
TOTAL	22.5	2115	832.5

Table 2.4 Reaction mix for IC reaction .

Reagents	µl /reaction	ul /94 reaction	ul /37reactions
Master Mix	10	940	370
IC Primer/probe mix (Vic_none)	1	94	37
Nuclease free Water	7	658	259
TOTAL	18	1692	666

Following preparation of the reaction mix the procedure was as follows. A separate plate was used for VP6 and IC reactions:

1. 22.5 µl (VP6) or 18 µl (IC) of reaction mix added to each well of a 96 well plate.
2. 2.5 µl cDNA (VP6) or 2 µl cDNA (IC) added to the plate
3. Plate sealed with adhesive cover
4. Plate spun for 5 seconds in plate centrifuge
5. Plate transferred to the ABI 7500 machine
6. Plate cycled at the following temperatures for each reaction:

Denaturation	95°C 2 minutes
40 cycles	95°C 15 seconds
	60°C 1 minute

For each run method, positive and negative controls (a positive sample and a negative sample that had undergone extraction and RT steps), a PCR positive control (cDNA containing the target) and RT and PCR negative controls (no template controls) were included. Results were analysed by reviewing positive and negative controls, adjusting the threshold above any background noise, and by reviewing the standard curve. The threshold was set at the beginning of the exponential curve in the linear graph, and the middle of the linear phase in the log graph. For the standard curve typically a correlation coefficient (R^2) of >0.9 and amplification efficiency of >80% and a minimum of 5 points within the assay linear range was considered adequate. Ct values of the standard curve were also checked against typical/expected values.

For the run to be accepted all negative controls had to be below the threshold, with no amplification, and positive controls had to demonstrate a Ct value<40 and a sigmoidal curve. Analysis was performed by laboratory technicians, reviewed and approved by the study PI (AB), and finally reviewed with MIG/KJ prior to accepting result as authorised. Assays were repeated when samples failed quality control (QC).

Table 2.5 Primer and probe composition for VP6 qRT-PCR

Primer/Probe	Sequence (5'-3')	Nucleotide position
VP6F	GAC GGV GCR ACT ACA TGG T	747-766(279)
VP6R	GTC CAA TTC ATN CCT GGT G	1126-1106 (279)
VP6P	^{FAM} CCA CCR AAY ATG ACR CCA GCN GTA ^{MGB}	

2.3.4.5 Rotavirus NSP3 qRT-PCR

This qRT-PCR used primers designed to amplify an 87 bp fragment of the non-structural protein 3 (NSP3) gene of group A rotaviruses. This assay was used for confirmatory purposes for samples which had Ct values >35<40 on VP6 PCR. The reaction mix was prepared at -20°C in the quantities described in Table 2.6. Primer and probe composition is described in Table 2.7

Table 2.6. Reaction mix for NSP3 PCR

Reagents	µl /reaction
Master Mix (with low Rox)	12.5
NSP3F (@20 pmol/ul)	0.4
NSP3R (@20pmol/ul)	0.4
NSP3 probe (FAM_MGB @ 20uM)	0.15
Nuclease free Water	9.05
TOTAL	22.5

Following preparation of the master mix, the following steps were followed

1. 22.5µl of NSP3 reaction mix was added to a 96 well plate
2. 2.5 µl of cDNA was added
3. The plate was sealed
4. Plate was spun in a plate centrifuge for 5 seconds
5. The plate was transferred to the ABI 7500 machine
6. The same cycling conditions as for VP6 PCR were used

Each run contained negative controls and low and medium concentration positive controls. Low and medium controls were made from stock generated from serial dilutions of a very concentrated sample. Medium controls had a target Ct value between 26 and 30, low controls were a 100x dilution of the medium control (Ct 34-37).

Table 2.7 Primer and probe composition for NSP3

Primer/Probe	**Sequence (5'-3')**	**Nucleotide position**
NSP3-F	ACC ATC TWC ACR TRA CCC TCT ATG AG	963-982
NSP3-R	GGT CAC ATA ACG CCC CTA TAG C	1,034-1049(280)
NSP3-Probe	$^{FAM-}$AGTTAAAAGCTAACACTGTCAAA ^{MGB}	995-1017(281)

2.3.4.6 Rotavirus NSP2 qRT-PCR

This qRT-PCR used primers designed to amplify a 281 bp fragment of the non-structural protein 2 (NSP2) gene of Rotarix™ vaccine rotavirus strain and as before included a target specific hydrolysis probe. The reaction mix was prepared at -20°C according to the quantities outlined in Table 2.8. Composition of primers and probes can be found in Table 2.9.

Table 2.8 Reaction mix for NSP2 PCR

Reagents	µl /reaction	ul /98 reactions
Master Mix (with low Rox)	12.5	1,225
NSP2F (@20 pmol/ul)	0.5	49
NSP2 R (@20pmol/ul)	0.5	49
NSP2 probe (FAM_MGB @ 20uM)	0.25	24.5
Nuclease free Water	9.25	906.5
TOTAL	23	2,254

Following preparation of the NSP2 reaction mix the following steps were followed:

1. 22.5 µl NSP2 reaction mix added to a 96 well plate
2. 2.5 µl cDNA added
3. Plate sealed.
4. Plate spun in plate centrifuge for 5 seconds
5. Plate transferred to the ABI 7500 machine.
6. Cycling conditions as for the VP6 PCR.

PCR negative controls, a Rotarix™ cDNA control, and a non-vaccine rotavirus positive cDNA control were included on each plate.

Table 2.9 Primer and probe composition for NSP2

Primer/Probe	Sequence (5'-3')	
RV1NSP2-F	GAA CTT CCT TGA ATA TAA GAT CAC ACT GA	546-574 (282)
RV1NSP2-R	TTG AAG ACG TAA ATG CAT ACC AAT TC	826-801 (282)
RV1NSP2-Probe	^{FAM}TCC AAT AGA TTG AAG TCA GTA ACG TTT CCA^{BHQ1}	782-753 (282)

2.3.4.7 Quality Control for RT-PCR

A series of quality control steps were incorporated at each stage of the extraction, RT and PCR process to ensure results were reliable.

Internal control (IC)

The internal control acted as a positive control for the extraction procedure. The IC is an RNA template of known quantity extracted along-side sample RNA and then detected on PCR. The IC PCR was run separately to the VP6 RT-PCR to avoid inhibition. Ct values of 26+/- 3 from the IC were considered acceptable. Analysis of IC plates followed the rules outlined above for VP6 PCR.

Positive control (PC)

One positive control was included per extraction batch. This was a known rotavirus positive stool sample with Ct value 30-37. Samples selected as controls were prepared as aliquots and used for as many extractions as possible to allow comparison of Ct values between batches. Ct values for positive controls were plotted to allow inspection for trends over time, and Ct values deviating >+/-3 Ct values from previous assays were considered unacceptable. The purpose of the positive control was to ensure that extraction, RT and amplification steps all occurred adequately.

Negative control (NC)

One negative control of sterile water was included per extraction batch in order to identify contamination in any of the steps.

cDNA control (cDNA)

This was added at the PCR stage to confirm that PCR master mix and reagents were prepared correctly. This was typically an aliquot of standard plasma.

PCR no template control (PCR NT)

This was a negative control included at the PCR stage to ensure no contamination occurred at the PCR stage.

2.3.4.8 Rotavirus Genotyping

G and P typing for rotavirus was conducted according to the method described by the European Rotavirus Network (EuroRotaNet, http://www.eurorota.net), a network of European laboratories collaborating to provide comprehensive rotavirus strain surveillance pre- and post- vaccine introduction, develop methods for rotavirus typing and respond to changes in molecular epidemiology associated with genetic drift(91). Genotyping was conducted on cDNA synthesised using random primers, as described above. It utilises a two-stage PCR with consensus and type-specific primers. Genotyping was performed through the diarrhoeal surveillance platform. Primary supervision and interpretation of results was conducted by MIG and KJ with support from LP and myself.

G-typing consensus PCR

First round PCR mix was prepared as described in table 2.10

Table 2.10 Reaction mix for G-type consensus PCR

Reagents	
10 x buffer II (Invitrogen)	4.5 ul
50mM $MgCl_2$	2.0 µl
dNTPs (10mM)	1.0 µl
Taq Polymerase (5U/ul) (Invitrogen)	0.2 µl
Primer VP7-F (20pmoles/ul)	1.0 µl
Primer VP7-R (20pmoles/ul)	1.0 µl
RNase-free H_20	35.3 µl
TOTAL	45.0 µl

Following preparation of the reaction mix 45 µl of mix was added to each PCR tube, and 5 µl cDNA added. PCR tubes were added to the thermocycler and cycled at the following temperatures:

94 °C	2 min	X1
94 °C	1 min	
52 °C	1 min	X35
72 °C	1 min	
72 °C	7 min	X1
15 °C	hold	

G-typing multiplex PCR

Second round PCR mix was prepared as outlined in Table 2.11

Table 2.11 Reaction mix for G-typing multiplex PCR

Reagents	
10 x buffer II (Invitrogen)	4.8 ul
50mM $MgCl_2$	2.5 µl
dNTPs (10mM)	1.0 µl
Taq Polymerase (5U/ul) (Invitrogen)	0.2 µl
Primer VP7-R (20pmoles/ul)	1.0 µl
Primer G1 (20pmoles/ul)	1.0 µl
Primer G2 (20pmoles/ul)	1.0 µl
Primer G3 (20pmoles/ul)	1.0 µl
Primer G4 (20pmoles/ul)	1.0 µl
Primer G8 (20pmoles/ul)	1.0 µl
Primer G9 (20pmoles/ul)	1.0 µl
Primer G10 (20pmoles/ul)	1.0 µl
Primer G12 (20pmoles/ul)	1.0 µl
RNase-free H_20	30.5 µl
TOTAL	48.0 µl

Following preparation of the reaction mix 48 µl of reaction mix was added to each PCR tube followed by 2 µl of first round product. PCR tubes were added to the thermocycler and cycled at the following temperatures:

94 °C	4 min	X1
94 °C	1 min	
42 °C	2 min	X30
72 °C	1 min	
72 °C	7 min	X1
15 °C	hold	

P-typing consensus PCR

First round PCR mix was prepared as outlined in Table 2.12

Table 2.12 Reaction mix for P-typing consensus PCR

Reagents	
10 x buffer II (Invitrogen)	4.5 ul
50mM $MgCl_2$	2.0 µl
dNTPs (10mM)	1.0 µl
Taq Polymerase (5U/ul) (Invitrogen)	0.2 µl
Primer VP4-F (20pmoles/ul)	1.0 µl
Primer VP4-R (20pmoles/ul)	1.0 µl
RNase-free H_20	35.8 µl
TOTAL	45.0 µl

Following preparation of the reaction mix, 45 µl of mix was added to each PCR tube, and 5 µl cDNA added. PCR tubes were added to the thermocycler and cycled at the following temperatures:

94 °C	2 min	X1
94 °C	1 min	
50 °C	1 min	X35
72 °C	1 min	
72 °C	7 min	X1
15 °C	hold	

P-typing multiplex PCR

Second round PCR mix was prepared as outlined in Table 2.13

Table 2.13 Reaction mix for P-typing multiplex PCR

Reagents	
10 x buffer II (Invitrogen)	4.8 ul
50mM $MgCl_2$	2.5 µl
dNTPs (10mM)	1.0 µl
Taq Polymerase (5U/ul) (Invitrogen)	0.2 µl
Primer VP4-F (20pmoles/ul)	1.0 µl
Primer P[4] (20pmoles/ul)	1.0 µl
Primer P[6] (20pmoles/ul)	1.0 µl
Primer P[8] (20pmoles/ul)	1.0 µl
Primer P[9] (20pmoles/ul)	1.0 µl
Primer P[10] (20pmoles/ul)	1.0 µl
Primer P[11] (20pmoles/ul)	1.0 µl
RNase-free H_20	30.5 µl
TOTAL	48.0 µl

48 µl of round mix was added to a 0.2ml tube, followed by 2 µl of first round product. PCR tubes were added to the thermocycler and cycled at the following

94 ^oC	4 min	X1
94 ^oC	1 min	
42 ^oC	2 min	X30
72 ^oC	1 min	
72 ^oC	7 min	X1
15 ^oC	hold	

Primer composition for G and P types are listed in Table 2.14

Agrose-gel electrophoresis

1.5g of UltraPure™ Agarose 100 was added to 100ml 1X Tris/Borate/EDTA (TBE) buffer to make 1.5% gel and melted in a microwave. Once melted, the gel was cooled to 45°C, and ethidium bromide added (at a concentration of 3.5g/L). The cooled gel was poured into a gel plate and fitted with two 22-28 slot combs. 10 µl of PCR product was added to 10 µl sample buffer in a micro-titre plate. Once set, the combs were removed. Samples were mixed with loading dye and 20 µl of either size marker (100bp ladder) or diluted sample added to each well. The gel plate was added to the gel tank, and TBE buffer added level with the gel. After running the products into the gel for 5 minutes using a voltage of 150V, the gel was flooded with TBE buffer such that it was fully submerged. Electrophoresis was then performed at a constant voltage between 5 and 8 V/cm until the samples had run the length of the gel. The gel was then placed into a UV transilluminator for visualisation.

Table 2.14 Primer composition for G and P typing (283)

Primer	Sequence (5'-3')*	Nucleotide Positions	Product Size
VP7-F	ATG TAT GGT ATT GAA TAT ACC AC	51-71	881bp
VP7-R	AAC TTG CCA CCA TTT TTT CC	914-932	881bp
G1	CAA GTA CTC AAA TCA ATG ATG G	314-335	618bp
G2	CAA TGA TAT TAA CAC ATT TTC TGT G	411-435	521bp
G3	ACG AAC TCA ACA CGA GAG G	250-269	682bp
G4	CGT TTC TGG TGA GGA GTT G	480-499	452bp
G8	TTR TCG CAC CAT TTG TGA AAT	176-198	756bp
G9	CTT GAT GTG ACT AYA AAT AC	757-776	179bp
G10	ATG TCA GAC TAC ARA TAC TGG	666-687	266bp
G12	GGT TAT GTA ATC CGA TGG ACG	548-567	396bp
VP4-F	TAT GCT CCA GTN AAT TGG	132-149	663bp
VP4-R	ATT GCA TTT CTT TCC ATA ATG	775-795	663bp
P[4]	CTA TTG TTA GAG GTT AGA GTC	474-494	483bp
P[6]	TGT TGA TTA GTT GGA TTC AA	259-278	267bp
P[8]	TCT ACT GGR TTR ACN TGC	339-356	345bp
P[9]	TGA GAC ATG CAA TTG GAC	385-402	391bp
P[10]	ATC ATA GTT AGT AGT CGG	575-594	583bp
P[11]	GTA AAC ATC CAG AAT GTG	305-323	312bp

***** R= A or G, N=A or T or G or C

2.3.5 Rotavirus Enzyme Immuno-assay (EIA)

EIA testing was performed on children presenting to QECH, as part of the ongoing diarrhoeal surveillance platform and in line with WHO recommendations for rotavirus surveillance(10). Stool

samples from children with acute gastroenteritis presenting to QECH were processed on arrival in the laboratory, and stored as 10% suspension in PBS at 4-7 degrees Celsius before batch testing weekly using a commercial EIA assay (Rotaclone, Meridian Bioscience, Cincinnati, Ohio) as per manufacturer's instructions.

2.3.6 Rotavirus Immunochromatographic (IC) rapid tests

IC testing was performed in real-time by nurses screening children for eligibility for the RotaRITE studies using Coris Rota-strip^{TM}. (Coris BioConcept, Gembloux, Belgium). Testing was performed according to manufacturer's instructions. All facilities used for recruitment had a small laboratory area for Malaria testing and this was used for stool testing for rotavirus. Results of the rapid test were recorded in the clinical notes regardless of whether the child was recruited into the RotaRITE study.

2.3.7 Rotavirus Serology methods

2.3.7.1 Anti-rotavirus IgA

Anti-rotavirus IgA titres were measured using a semi-quantitative sandwich ELISA developed by the Wellcome Trust Research Laboratory Christian Medical College, Vellore (SOP no W/ASA/25 V3.0 for Quantitation of Anti-Rotavirus IgA by ELISA) and based on a method developed by R Ward(284). Standards and controls were donated from Wellcome Trust Research Laboratory Christian Medical College, Vellore. 96 well plates were coated with rabbit anti-rotavirus hyperimmune serum and incubated over night with WC3 rotavirus containing cell culture lysate (MA104). 4x2 serial dilutions of sera (4X2) were then added and a biotinylated rabbit anti-human IgA (Jackson ImmunoResearch Lab, US), avidin-biotin-peroxidase complex (Vecastain ABC kit; Vector) and a peroxidase substrate (o-Phenylenediaminedihydrochloride; Sigma) used for anti-rotavirus IgA detection. Each run included a standard curve (serial dilutions of control plasma (8x2)) and positive and negative controls.

Results were calculated on a minimum of two values per sample with a coefficient of variation (CV) < 20% and were expressed as geometric mean titres (IU/ml IgA). Trimmed geometric means were used where a CV of <20% could not be obtained after repeat testing. Results were classified as zero if below the lower limit of detection.

2.4 Statistical Methods

Statistical methods are described in detail in each section. Analysis was conducted using Stata version 14.2 (StataCorp, USA), GraphPad Prism 6 (GraphPad Software Inc, USA), and R 3.0.2 (R Foundation for Statistical Computing, Austria). Missing data was rare, and unless otherwise specified missing variables were managed by exclusion from analysis.

2.5 Data Management

Data were collected and stored in accordance with international Good Clinical Practice (GCP) guidelines. At the point of recruitment each subject was given a unique identifier. For the RotaRITE studies, and for surveillance data collected from 18th January 2015 onwards, source data were captured using pre-coded paper case record forms (CRFs). Intelligent Character Recognition scanning software (Teleform) was used to convert data into electronic form. Data were stored in a SQL database on the secure MLW server, and extracted directly into Stata. Prior to January 2015 data capture for the diarrhoeal surveillance programme was conducted by direct data entry into a redcap database.

The data management systems were designed by AB, with support from the data department at MLW. Data discrepancies and queries were identified and recorded using specifically designed algorithms and automated data checks, and were manually checked against the study CRF. Following data cleaning data manipulation and database construction was conducted by me using Stata 14.2.

2.6 Ethical Considerations

Ethical approval for VacSurv was obtained from the National Health Sciences Research Committee, Lilongwe, Malawi (867), and by the University of Liverpool Research Ethics Committee (000490). Ethical approval for RotaRITE Transmission Epidemiology was obtained from the Malawi College of Medicine Research Ethics Committee (P.09/14/1623) and University of Liverpool Research Ethics Committee (RETH000757). Sponsorship for the RotaRITE Transmission Epidemiology was obtained from University of Liverpool (UoL001070).

RESULTS

SECTION A

Chapter 3. Estimating the incidence of rotavirus infection in children from India and Malawi using serial anti-rotavirus IgA titres

3.1 Introduction

Rotavirus has been, prior to the introduction of rotavirus vaccines, the commonest cause of AGE in children worldwide, responsible for enormous morbidity and substantial mortality(58,285–288). As a result of a cohesive international effort to develop and license vaccines against rotavirus, there are currently two live, oral vaccines against rotavirus disease which are globally licensed, the monovalent RV1 and the pentavalent RV5(2). Both of these vaccines showed high efficacy in clinical trials in high income settings (98-100%(139,140)), but notably lower efficacy in low and middle income settings. Despite this, in view of the extent of the disease burden in lower income countries the WHO recommended routine introduction of vaccine into such settings as a priority in 2009, and rotavirus vaccine has now been introduced in over 35 GAVI eligible countries(289). Post implementation vaccine effectiveness data has begun to emerge from LIC, and while vaccine effectiveness appears to be higher than observed efficacy in clinical trials, it remains lower than reported from high income settings. Section A of this thesis uses existing datasets to address questions regarding population rotavirus transmission in the context of reduced vaccine effectiveness in LIC. This chapter investigates a novel approach to estimating force of infection in young infants using serological data, the next chapter will use surveillance data to investigate for rotavirus vaccine indirect effects in Malawi, a LIC.

Accurate descriptions of force of rotavirus infection are important given the sub-optimal vaccine effects reported from lower income settings. Firstly, force of infection, or the rate at which susceptible individuals acquire infection(252), is one possible contributing factor to reduced vaccine effectiveness. Force of infection is typically higher in low income settings, as evidenced by the early onset of rotavirus disease in such countries(144). High force of infection may result in high titres of circulating maternal antibodies against rotavirus, and trans-placental transfer of maternal IgG may interfere with the infant's ability to develop an immune response to the vaccine. In keeping with this, a negative correlation has been demonstrated between high maternal IgG titres and infant response to vaccine measured by anti-rotavirus IgA titres(143,290,291). Similarly, anti-rotavirus IgA excreted in breast milk from women in LIC has been shown to contain higher titres of IgA, and to demonstrate greater neutralising ability against rotavirus, than breast milk from women in HIC(292,293).

High force of infection may also lead to epidemiological artefact in the measurement of vaccine effects. For examples, measurement of vaccine efficacy relies on evaluation of the difference in infection rates between vaccinated infants and unvaccinated control infants, and rotavirus vaccine is

designed to mimic natural immunity which provides incremental protection against severe disease. In settings with a high force of infection natural exposure and subsequent acquisition of immunity in the control arm may result in similarities in the rate of infection between case and control groups, and an apparent reduction in measured vaccine efficacy(145). Describing differences in patterns of force of infection between populations is therefore important to improve understanding of variation in vaccine performance.

Secondly, describing population level changes in incidence before and after vaccine implementation is an important means of measuring vaccine impact, particularly for diseases such as rotavirus where the spectrum of disease is wide and not all cases will present to sentinel surveillance sites. It can also capture rotavirus vaccine indirect effects, or reductions in disease in unvaccinated sections of the population. Evaluations of impact of the vaccine on rotavirus transmission at the population level contribute to accurate estimations of cost effectiveness, which is important to inform vaccine policy. Finally, understanding the timing of peak rotavirus incidence in children could help inform vaccine scheduling, which is one potential mechanism to improve vaccine performance(294).

Obtaining accurate estimates of rotavirus incidence is challenging however, as asymptomatic infection is common, and shedding of virus in stool is transient(38). Several cohort studies have provided invaluable data on rotavirus incidence in infants and children with intensive monitoring and collection of serial stool and serum samples(38,62,295), but such studies are expensive and logistically challenging to be conducted on a wide scale. Sero-surveys have the potential to be a pragmatic and cost-effective means to provide useful information on population level incidence. Serum anti-rotavirus IgA has been shown to reflect intestinal IgA, which is thought to be important in immunity against rotavirus(296,297). Serum IgA rises in response to rotavirus infection, and in response to rotavirus vaccination(63,64,66). IgA seroconversion rates post vaccination have been shown to correlate with vaccine efficacy, and anti-rotavirus IgA titres to correlate with protection against severe wild-type disease(63,298).

Although sero-surveys are an attractive option, making meaningful inferences from changes in antibody titre is not straight-forward. Serological assays have considerable internal and external heterogeneity, and natural fluctuations in antibody levels within subjects may be physiological and not necessarily meaningful. Traditionally, fold increase in titre or antibody levels above a certain threshold have been taken to demonstrate seroconversion(38,284), but both of these methods are prone to misclassification(299,300). As an alternative approach we used mixture models as a quantitative analytical method to evaluate changes in anti-rotavirus IgA titres over time and estimate rotavirus incidence in infants and young children from two different low income, unvaccinated

populations: an urban slum in Vellore, Southern India, and a rural setting in Karonga, Northern Malawi. Mixture models (two component Gaussian mixture distributions) have been used to interpret serological data for other pathogens(300), but to our knowledge have not previously been used for rotavirus. They have potential advantages over more traditional methods of analysing serological data as they evaluate data probabilistically, and offer a visual interpretation of the data. In this study we compared results from mixture model to findings from more commonly used techniques of fold increase and use of a pre-defined cut-off to evaluate change in antibody titres. Patterns of incidence over time were compared between the two settings in order to better understand potential differences in rotavirus transmission in different environments.

3.2 Methods

3.2.1 Objectives

1. To describe incidence of rotavirus infection in young infants from Vellore, India, using mixture models

2. To describe incidence of rotavirus infection in young infants from Karonga, Malawi, using mixture models

3. To compare estimates of rotavirus incidence derived from mixture models to those derived using alternative methods of interpreting serological data (fold increase and a pre-defined cut off)

3.2.2 Study design

This study used serum samples previously collected from infants and children enrolled into birth cohorts in two low and middle income different locations.

3.2.3 Study site

The birth cohorts were located in an urban slum in Vellore, Southern India, a LMIC; and Karonga, a rural setting in Northern Malawi, a LIC (Fig 3.1). Both are tropical countries, with similar altitudes (204 metres above sea-level for Vellore, 529 metres for Karonga). Average yearly temperature is 30^{o}C in Karonga and 28^{o}C for Vellore(119,301). Both have an annual monsoon. Neither population had introduced rotavirus vaccine at the time of study.

3.2.4 Study population

Both studies recruited newborn infants and followed then up prospectively.

3.2.5 Study procedures

3.2.5.1 Indian birth cohort.

The Vellore cohort was designed to investigate acquisition of natural protective immunity to rotavirus and recruited infants from within a Demographic Health Surveillance site located within three adjacent slum regions in Vellore, with a population density estimated at 17,000/km^2. During follow up, households were visited twice weekly to collect symptom information for the enrolled infant and other household members. Stool samples were collected fortnightly, and serum samples were collected from the recruited infant at birth and then every six months. Stool samples were tested for

rotavirus using EIA (Rotavirus IDEIA, Dako), and RT-PCR. A detailed description of this cohort has been previously published(38).

3.2.5.2 Malawi birth cohort.

The Karonga birth cohort was located within the Karonga Health and Demographic Surveillance System (HDSS) in northern Malawi, a rural region, with a population density of approximately 264/km^2. The birth cohort was designed to investigate pneumococcal carriage in HIV exposed mothers and their infants(302,303). Infants were recruited between January 2009 and December 2010. Serum samples were collected at 6, 26 and 52 weeks of life.

Figure 3.1 Location of study sites. A) Karonga, Malawi B) Vellore, India

Map data ©2017 Google

3.2.6 Laboratory methods

Anti-rotavirus IgA antibodies were measured using a standard sandwich ELISA(304). Standard Operating Procedures (SOPs), as well as the IgA standards and control plasma used were the same across both sites, the only difference being that the Vellore site used 2 x 10 fold dilution of sera and

Karonga used 4 x 2 fold dilutions. The assay methods are described in detail in chapter 2.3.7.1, page 100. Serology for the India cohort was performed by the study team in Vellore.

3.2.7 Statistical Analysis

Statistical analysis comprised five stages, which are outlined in Fig. 3.2.

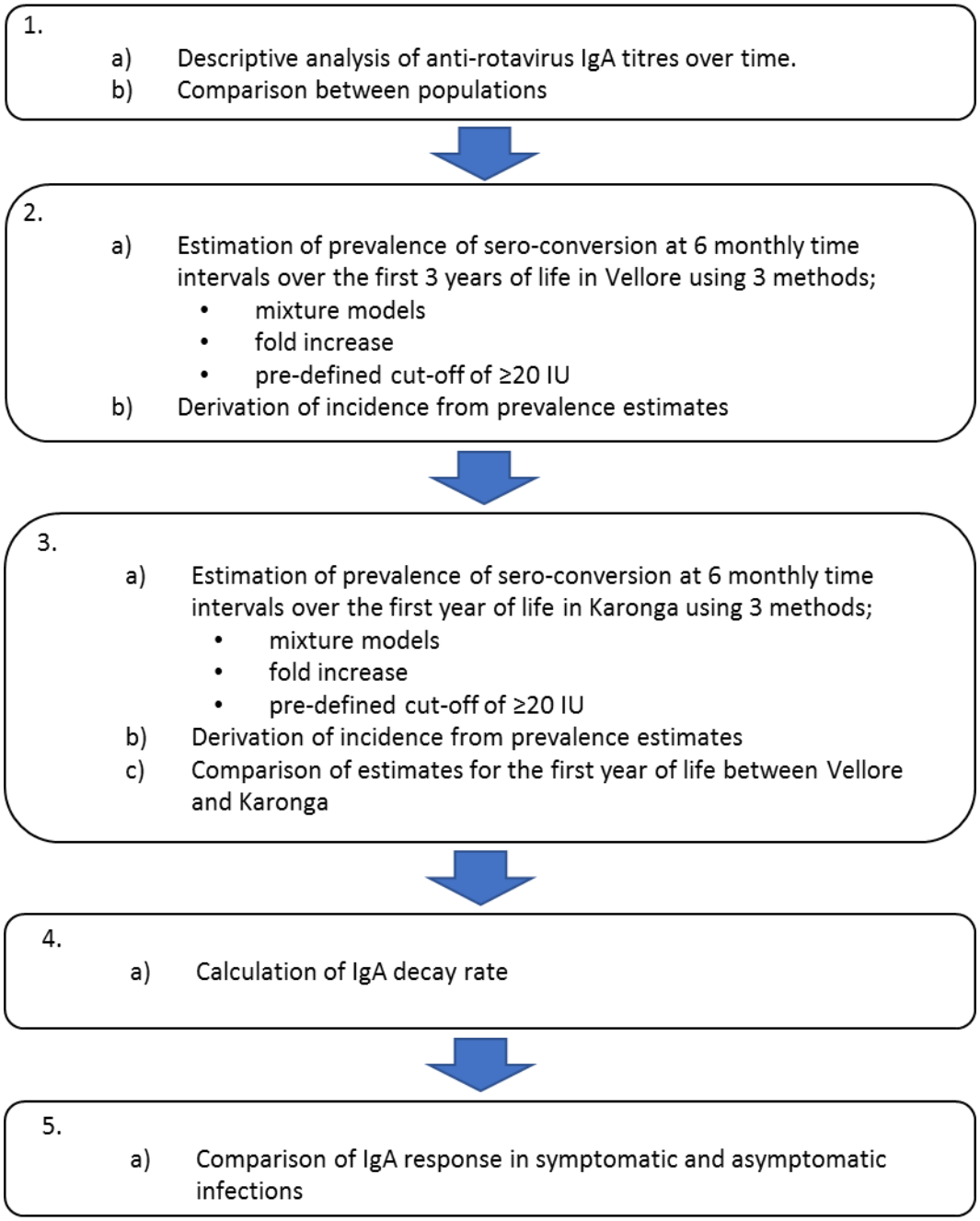


Figure 3.2. Outline of statistical analysis.

3.2.7.1 Descriptive analysis

Descriptive analyses consisted of comparing median titres between time points within and across populations. Independent and paired medians were compared using sign-rank and rank-sum tests respectively. Chi-squared for independent proportions was used to compare the proportion of children demonstrating seroconversion between populations, where seroconversion was defined as titre ≥20IU/ml(284). The cut-off of 20IU/ml to define sero-conversion has been used through multiple vaccine immunogenicity and efficacy studies, particularly in relation to RV1.

3.2.7.2 Estimating prevalence of sero-conversion over the first 3 years of life time in Vellore

Firstly, prevalence of seroconversion was estimated using mixture models. Mixture models refer to two component Gaussian mixture distributions, where one component is assumed to correspond to uninfected individuals and one, with larger values, to seroconverted (presumed infected) individuals. These were used to estimate the prevalence of seroconversion in Vellore at 6 monthly time intervals, based on increment in log transformed antibody titres between time points. Prevalence refers to the proportion of samples assigned to the positive or "infected" distribution(300,305).

Antibody titres were log transformed after adding one to the value of each titre to allow log transformation of zero values. Increment in log transformed titres was then calculated (i.e. 26-6 weeks [d1], 52-26 weeks [d2], 78-52 weeks [d3], 104-78 weeks [d4], 130 and 104 weeks [d5] and 156-132 weeks [d6]). Histograms of the difference in IgA titres between time points showed a bimodal distribution, but also identified large numbers of zero values (representing no change in antibody titre) for each time point. These did not fit a Gaussian distribution, and were therefore removed from the dataset prior to fitting the models. Examples of these distributions are shown in Fig. 3.3. Mixture models were then fit to the increment in log transformed titres between each of the time points. As the zero increment values clearly represented no evidence of re-infection, they were added back into the uninfected component for calculations of prevalence and incidence. Bootstrap confidence bounds were calculated around the model derived parameter estimate for prevalence of seroconversion.

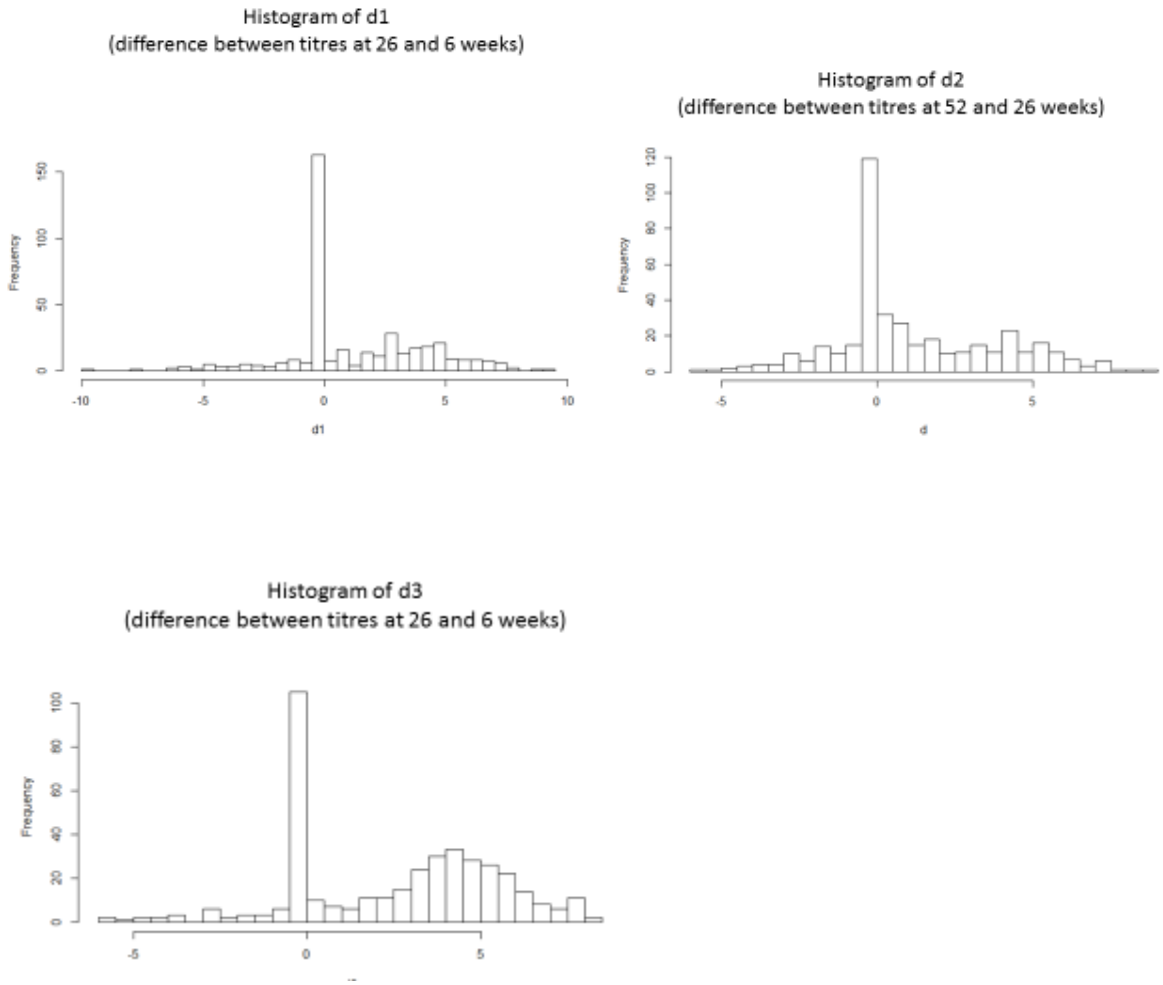


Figure 3.3. Example histograms of differences in IgA titres between time points (d1, d2, and d3 for titres at 26-6 weeks, 52-26 weeks and 52-6 weeks respectively) showing large numbers of zero values. Plots shown for combined Vellore and Karonga data.

3.2.7.3 Estimating seroconversion using fold increase and a pre-defined cut-off

To evaluate the use of mixture models, seroconversion was also calculated using two alternative definitions of seroconversion which are commonly used in the literature and in clinical trials of vaccine immunogenicity; fold increase and a pre-defined cut-off of anti-rotavirus IgA titres ≥ 20IU. For calculation of fold increase 0.1 was added to each assay result (to allow calculation of fold increase for zero values), and seroconversion was defined as a three-fold or greater rise between time points. For the cut-off of ≥20IU, sero-positivity was defined as IgA titres ≥ 20IU and becoming seropositive between time points was considered seroconversion.

3.2.7.4 Estimating incidence from prevalence estimates

Rotavirus infection incidence λ, during the interval τ between each time point was calculated based on a log-linear fit using the formula below, where *p* corresponds to the prevalence of sero-conversion(306). For mixture models this refers to the bootstrap estimate of mean prevalence:

$$\lambda = \frac{-\ln(1-p)}{\tau}$$

3.2.7.5 Estimating prevalence of sero-conversion and incidence in Karonga

To compare exposure to rotavirus infection in infancy between the Vellore and Karonga populations, mixture models were fit to the increment in log transformed titres between 6 and 26 weeks, 26 and 52 and 6 and 52 weeks in Karonga, and the prevalence of seroconversion and derived incidence for each time point compared to that demonstrated in Vellore. Prevalence and incidence of seroconversion were also calculated using fold increase and a cut-off value, as outlined above. To further validate findings, the difference between increments was calculated for each location (i.e [d2]-[d1]), and the mean value compared between locations using a two-sample t-test.

3.2.7.6 Calculation of IgA decay rate

To evaluate the likelihood of capturing repeated infection episodes using mixture models, a decay rate was calculated for anti-rotavirus IgA using a subset of children from the Vellore dataset. Serum samples were included if a child had a confirmed rotavirus infection in the first 6 months of life (asymptomatic or symptomatic stool infection, or a 3 fold increase in IgA titres). Children who had a repeat infection between 26 and 52 weeks (defined using stool or serology) were excluded. 87 children were identified who had a stool or serologically confirmed rotavirus infection in the first 26 weeks of life, and no evidence of re-infection between 26 and 52 weeks. Antibody decay was then calculated based on the log of the fold increase in titres between 26 and 52 weeks. The mean fold increase was estimated on log transformed titres, and then exponentiated.

3.2.7.7 Mixture models for asymptomatic and symptomatic infection

To gain further understanding of the IgA response to infection, an attempt was made to fit mixture models to the Vellore dataset for children between 6 and 26 weeks of life with known asymptomatic or symptomatic infections, defined as detectable rotavirus in surveillance or diarrhoeal stool samples. Only children with single infections during the time period were included. This analysis was under-powered as very few children had isolated asymptomatic infections, even in the first 6 months of life.

3.2.8 Ethics

Ethical approval for Vellore was obtained from the institutional review boards of Christian Medical College, Vellore; London School of Hygiene and Tropical Medicine, London; and Baylor College of Medicine, Houston. Ethical approval Ethical for Karonga was obtained from the National Health Sciences Research Committee in Malawi (protocol 490) and the London School of Hygiene and Tropical Medicine ethics committee (protocol 5345).

3.3 Results

A total of 452 newborns were enrolled into the Vellore birth cohort between 2002 and 2006 and follow up was completed in 373 infants. Households had a median size of five (range 2-11). Samples were available from the 373 infants who completed follow up(64). The Karonga birth cohort recruited 190 infants between November 2008 and November 2010, 28% of whom (54) were exposed to HIV. 112 infants had complete sets of three serum samples. Samples were chosen for anti-rotavirus IgA analysis if they contained more than 100µl of serum. After this selection process, 198 samples were available from 66 children(303).

3.3.1 Descriptive analysis

Serum IgA titres rose incrementally in both Vellore and Karonga, with significant rises in median IgA titres between time points (Fig. 3.4).

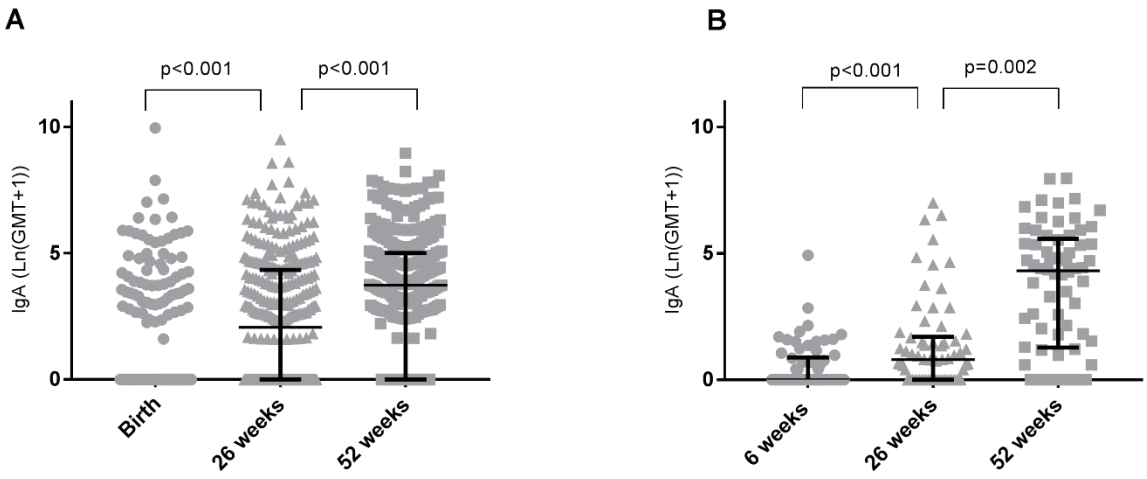


Figure 3.4. Serum IgA titres over time in Vellore (A) and Karonga (B). Error bars represent median and IQR. P values represent sign-rank tests for paired medians. Panel (B) modified from a figure submitted as part of an MRes report for A Bennett

The proportion of children with anti-rotavirus IgA titres ≥20IU/ml was greater in Vellore than Karonga at 6 (15.43% vs 1.52%, chi-squared test p=0.002) and 26 (37.78% vs 13.64 %, chi-squared p<0.001) weeks of life, but there was no significant difference between the two populations at 52 weeks of life (61.13% vs 60.61%, chi squared p=0.937) (Fig. 3.5)

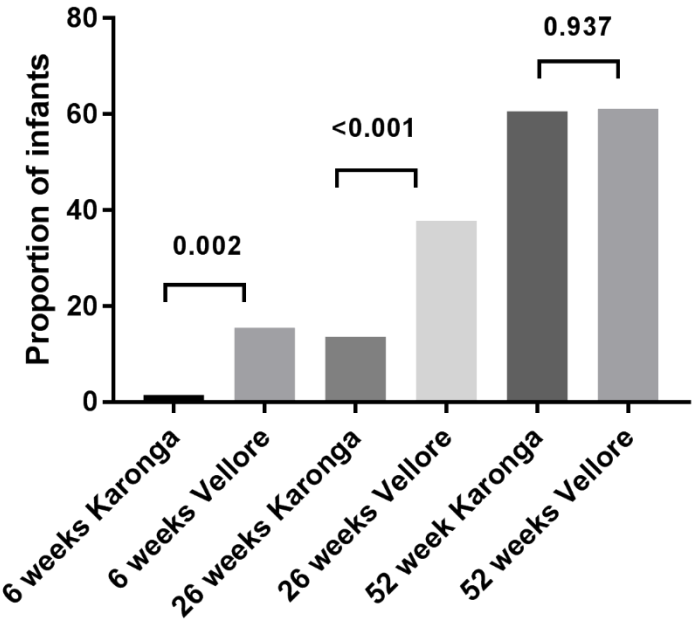


Figure. 3.5. Proportion of infants with anti-rotavirus IgA titres ≥20IU/ml in Vellore and Karonga. P values represent chi-squared tests for difference in independent proportions.

3.3.2 Patterns of rotavirus infection in first 3 years of life

Mixture models fit to the Vellore data over 3 years showed an initial high frequency of rotavirus infection, with a prevalence of seroconversion of 0.41 (95% CI 0.27 – 0.56) between birth and 6 months, which declined within each subsequent time interval (Fig 3.6 and Table 3.1).

Table 3.1. Parameter estimates from mixture models for rotavirus infection in Vellore and Karonga.

	Mean 1* ('uninfected')	SD* 1	Mean 2 ('infected')	SD 2	Mean prevalence of seroconversion** (95% CI)	Incidence rate of rotavirus infection† (95% CI)
6 monthly time intervals						
Vellore						
0-26 weeks	-2.44	2.68	3.97	1.86	0.41 (0.27-0.56)	1.05 (0.64-1.64)
26-52 weeks	0.11	2.08	5.02	1.42	0.20 (0.08-0.40)	0.44 (0.17-1.02)
52-78 weeks	0.26	1.84	4.69	1.77	0.18 (0.02-0.72)	0.39 (0.04-2.57)
78-104 weeks	-0.08	1.36	4.52	1.43	0.13 (0.02-0.54)	0.29 (0.04-1.57)
104-130weeks	-0.12	1.31	3.84	1.41	0.11 (0.02-0.47)	0.24 (0.04-1.29)
130-156 weeks	-0.17	1.69	5.14	1.36	0.05 (0.00-0.78)	0.10 (0.00-3.04)
Karonga						
6-26 weeks	0.40	1.45	4.87	1.22	0.15 (0.04-0.44)	0.34 (0.08-1.17)
26-52 weeks	0.08	1.31	4.48	1.35	0.50 (0.33-0.68)	1.41 (0.79-2.29)
12 monthly time intervals						
Vellore						
0-52 weeks	-0.35	2.62	4.62	1.53	0.55 (0.48-0.62)	0.80 (0.65-0.97)
Karonga						
6-52 weeks	-0.22	0.59	4.26	1.82	0.71 (0.42-0.90)	1.25 (0.54-2.28)

Data from Vellore for 156 weeks, from Karonga for 52 weeks. *Where mean 1 and SD1 refer to mean and standard deviation (SD) for distribution 1 (uninfected), and mean 2 and SD2 to mean and standard deviation for distribution 2 ("infected"). **Mean prevalence and confidence intervals (CI) derived from bootstrap estimates † Incidence rate derived from mean prevalence using formula stated previously. Incidence rate in episodes per child year.

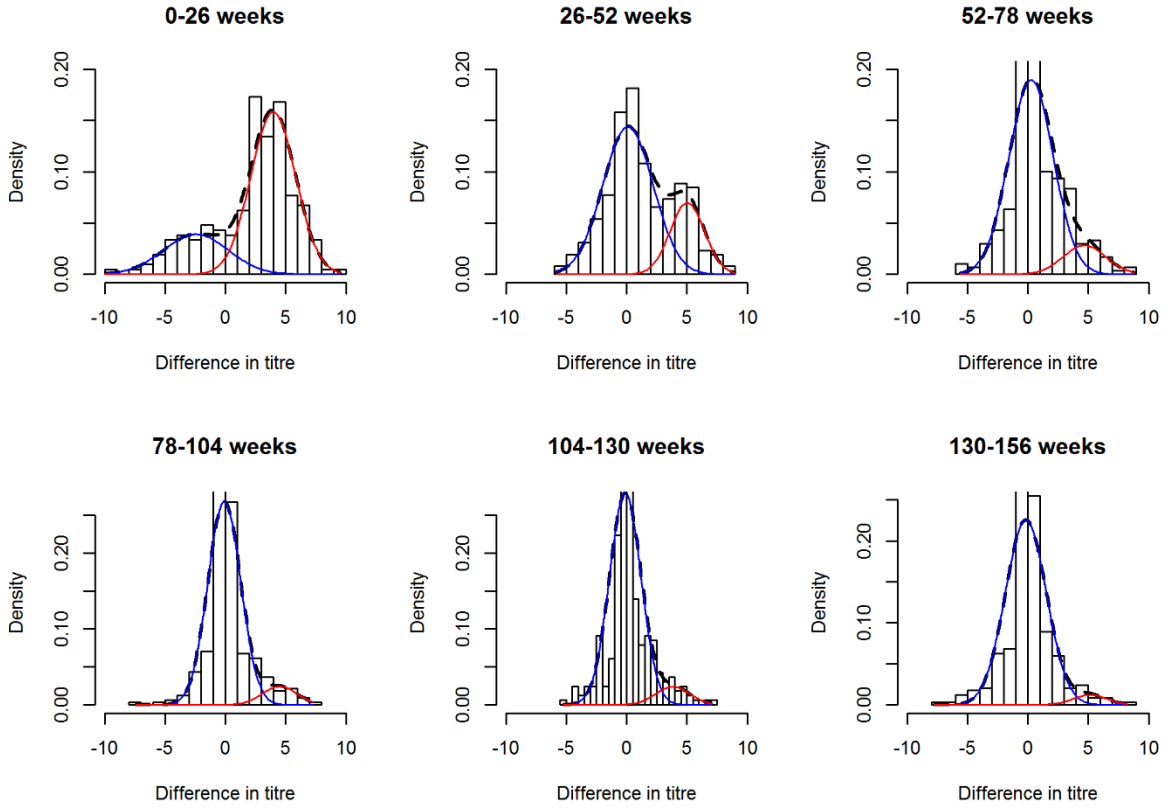


Figure 3.6 Mixture models showing positive ("infected") and negative ("uninfected") distributions for increment in log transformed anti-rotavirus IgA titres between 6 month time points in Vellore. Red lines indicate positive distributions and blue lines negative distributions.

3.3.3 Comparison of patterns of rotavirus infection in first year of life between Vellore and Karonga

Fitting mixture models to the Karonga data demonstrated that the incidence of rotavirus infection varied by time and between the two populations. Between 6 weeks and 26 weeks, the incidence of infection in Karonga was lower than that observed in Vellore with 0.34 episodes/child year (95% CI 0.08-1.17) compared to 1.05 episodes/child year (95% CI 0.64-1.64) (Fig 3.7 and Table 3.1). In comparison, incidence was considerably higher in Karonga between 26 and 52 weeks than in Vellore (1.41 episodes/child year [0.79-2.29] vs 0.44 episodes/child year [0.17-1.02]) (Fig 3.7 and Table 3.1). There was no clear difference between the two populations when incidence was calculated between 6 and 52 weeks (1.25 episodes per child year [0.54-2.28] in Karonga, versus 0.80 [0.65-0.97] in Vellore).

Consequent to the high level of antibodies in the first six months of life, and a lower relative increase in titres in the second six months of life in Vellore, the mean difference between change in titres ([d2]-[d1]) was significantly smaller in Vellore compared to Karonga (-0.35 in Vellore and 1.45 in Karonga, two sample t-test p=0.004).

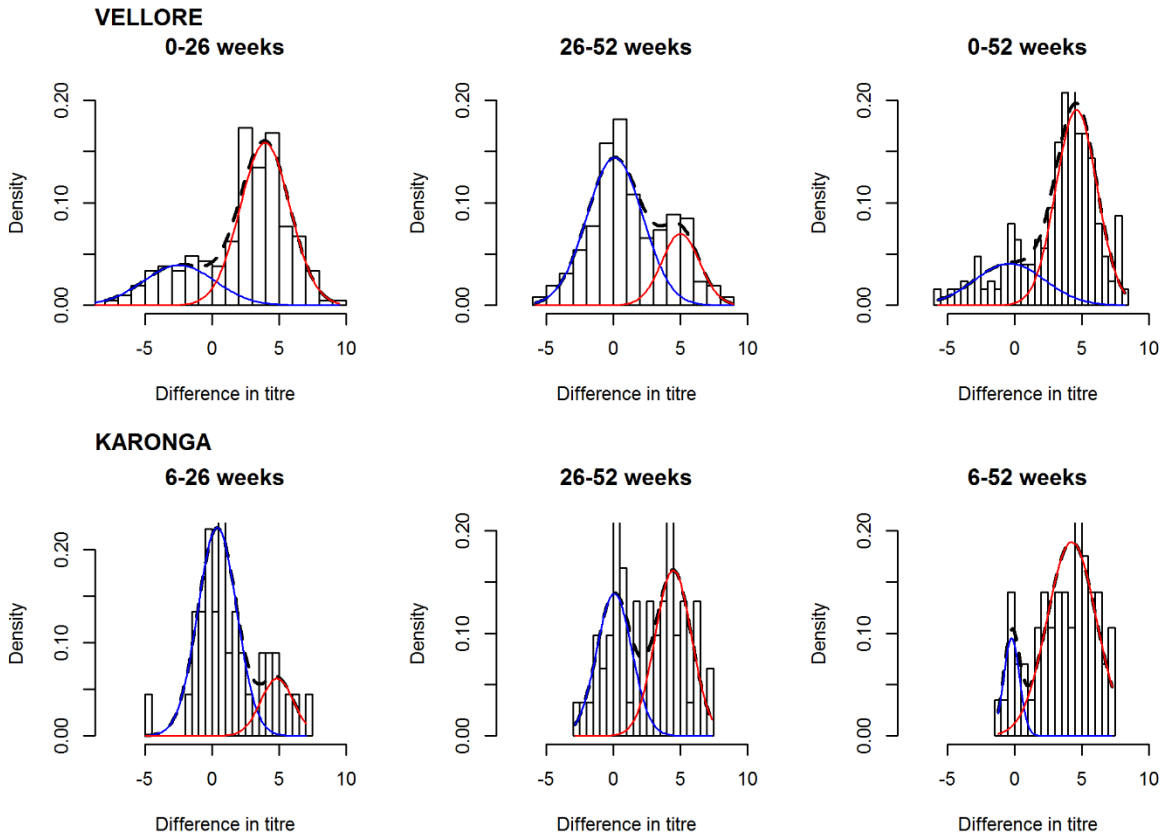


Figure 3.7. Mixture models showing positive ("infected") and negative ("uninfected") distributions for increment in log transformed anti-rotavirus IgA titres for the first year of life in Karonga and Vellore. Red lines indicate positive distributions and blue lines negative distributions.

Overall, estimates of incidence were comparable using the mixture models and the two alternative definitions of sero-conversion (Table 3.2). The notable exception was for estimating incidence between 6 and 26 weeks, when using fold increase resulted in a substantially higher estimate in Karonga (1.10 episodes/child year [95% CI 0.72-1.58]) than using mixture models or IgA titres >= 20 IU (0.34 episodes/child year [95% CI 0.08-1.17] and 0.29 episodes/child year [95% CI 0.11-0.50], respectively). Apart from this, all three methods showed higher incidence of seroconversion in Vellore compared to Karonga in the first 6 months of life, and higher incidence in Karonga compared to Vellore between 26 and 52 weeks.

Table 3.2. Prevalence of and incidence of rotavirus seroconversion between 0/6 and 26 weeks, 26 and 52 and 0/6 and 52 weeks for Vellore and Karonga using mixture models, fold increase and IgA titres>=20IU.

	Mixture model		Fold increase		IgA titres>=20IU	
	Prevalence*	Incidence †	Prevalence	Incidence †	Prevalence	Incidence †
0/6 weeks to 26 weeks						
Vellore	0.41 (0.27-0.56)	1.05 (0.64-1.64)	0.44 (0.39-0.49)	1.16 (0.98-1.36)	0.31 (0.26-0.36)	0.73 (0.60-0.88)
Karonga	0.15 (0.04-0.44)	0.34 (0.08-1.17)	0.42 (0.30-0.55)	1.10 (0.72-1.58)	0.14 (0.05-0.22)	0.29 (0.11-0.50)
26 to 52 weeks						
Vellore	0.20 (0.08-0.40)	0.44 (0.17-1.02)	0.35 (0.30-0.40)	0.87 (0.72-1.04)	0.28 (0.23-0.33)	0.66 (0.53-0.80)
Karonga	0.50 (0.33-0.68)	1.41 (0.79-2.29)	0.61 (0.49-0.73)	1.86 (1.33-2.60)	0.50 (0.38-0.62)	1.39 (0.94-1.96)
0/6 weeks to 52 weeks						
Vellore	0.55 (0.48-0.62)	0.80 (0.65-0.97)	0.60 (0.54-0.65)	0.91 (0.79-1.05)	0.50 (0.45-0.56)	0.70 (0.60-0.82)
Karonga	0.71 (0.42-0.90)	1.25 (0.54-2.28)	0.74 (0.63-0.85)	1.36 (1.01-1.90)	0.59 (0.47-0.71)	0.89 (0.63-1.25)

*Mean prevalence and confidence intervals (CI) derived from bootstrap estimates † Incidence rate derived from prevalence estimate using formula stated previously. Incidence rate in episodes per child year.

3.3.4 Anti-rotavirus IgA antibody decay

Based on the log of fold increase in titre, anti-rotavirus IgA titres showed a relatively rapid decay with a mean fold increase of 0.09 fold/year (i.e. > 10X reduction in antibody titres) following an initial infection (Fig 3.8).

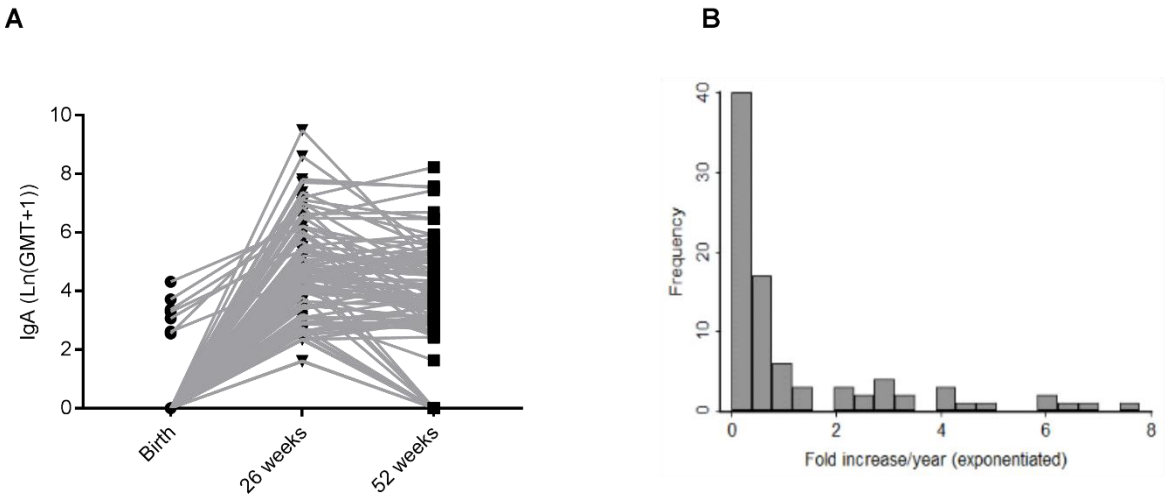


Figure 3.8. Anti-rotavirus IgA decay rates. A) Log transformed titres at 0, 26 and 52 weeks for 87 children included in IgA decay analysis. B) Fold increase in titres per year for the same children. Titres log transformed then exponentiated.

3.3.5 Mixture models for asymptomatic and symptomatic infection

Fitting mixture models to the Vellore dataset for children who had symptomatic compared to asymptomatic infection was limited by the sparse data for children with single asymptomatic infections. Despite this, prevalence of seroconversion appeared higher in children with symptomatic disease compared to asymptomatic infection (Fig. 3.9)

a) b)

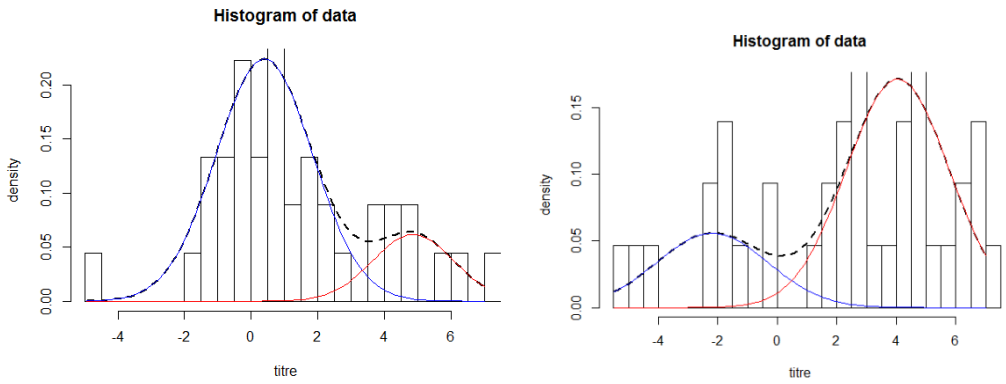


Figure 3.9 Mixture models for children who had a) a single asymptomatic infection and b) a single symptomatic infection between 6 and 26 weeks of life.

3.4. Discussion

It is crucial that we increase our understanding of patterns in force of infection for rotavirus in different populations as heterogeneity in incidence rates is one potential explanatory factor in the disparity in vaccine effects observed between HIC and low and middle income countries. These data presented demonstrate intriguing differences in the pattern of exposure to rotavirus infection in early life between populations in two distinct low and middle income countries, with children from Vellore infected with rotavirus at a younger age than children from Karonga, Malawi. Although the confidence limits for these estimates of prevalence and incidence are wide, the validity of these findings is corroborated in several ways including the consistency of the findings regardless of the definition of seroconversion; the estimate of annual incidence in the first year of life which is consistent with incidence estimates in other populations for the same age period(38); and a significant difference in mean titre increment from the first six to the second six months of life between populations.

The incidence patterns described were broadly comparable for all 3 methods used to define seroconversion. The major exception was for prevalence of seroconversion defined by fold increase in the first six months of life in Karonga. Here, substantially more children were identified as having seroconverted using fold increase (42%) than either mixture models (15%) or a pre-defined cut-off (14%). One possible explanation for this is mathematic artefact, as a very small proportion of children had IgA titres ≥20 IU at the time of first sample in Karonga, making it easier to achieve a significant fold increase(299). This is corroborated by a sensitivity analysis where the value of two was added to each value (results not shown). The value of two was selected as it is half the lower limit of detection in the original assay(284). When the higher value was added to zeros there was a reduction in the rate of sero-conversion as defined by fold increase, and in the subsequent incidence estimate. It is reassuring that the use of a cut-off of 20IU provides similar estimates of the prevalence of seroconversion to the mixture models, and argues against any major misclassification of sero-status in previous rotavirus sero-response studies.

The timing of peak incidence of seroconversion between populations, with an earlier peak in Vellore, suggests that force of rotavirus infection is greater in Vellore compared to Karonga. This is particularly intriguing because both are low income settings. LIC are typically associated with high force of rotavirus infection compared to HIC(144,210), which is likely to be a function of several different variables associated with poverty. These may include such factors as a high birth rate and consequently a larger pool of young children, thought to be crucial for introduction of rotavirus infection, in the community(245). Similarly over-crowding, with multiple people living and sleeping in the same space, is likely to play a role in propagating transmission(269), particularly if combined with

lack of reliable access to clean water and poor sanitation(307). Host factors may also play a role as it has been shown that repeated episodes of symptomatic rotavirus gastroenteritis may be required to provide protection in LIC, and given that symptomatic disease is thought to be the primary mediator of transmission, this could also contribute to high force of infection(38). Aside from factors relating to poverty, differences in climate or weather patterns such as humidity, differences in temperature, or flooding may also contribute(210,270,271,308).

Variation in any of the factors listed above could potentially explain why the force of rotavirus infection in young children is greater in Vellore compared to Karonga, but it seems likely that the striking differences in population density plays a significant role, with Vellore an urban slum region with a population density of 17000/km^2, in contrast to the rural Karonga district, which has a population density of approximately 264/km^2(302,309). The potential role of population density is substantiated by a recent study from Dhaka, Bangladesh which reported rotavirus incidence rates in the densely populated core of the city of almost 3 times those observed in the less densely populated peripheries, or a rural region of Bangladesh(270). In terms of other potential contributing factors, the observed difference is unlikely to be explained by birth rate, which is higher in Karonga compared to Vellore (47/1000 person years in 2005 vs 18/1000 person years between 1995 and 2003), or by household size, the median of which is 5 for both populations. Climate is also similar between the populations and it thus seems unlikely to be a major contributor, whilst acknowledging that there may be subtle differences that we do not have capacity to account for in this analysis. It is intriguing that there is a significant proportion of children who have detectable IgA at birth in Vellore, with approximately 15% having IgA titres ≥20 IU. Transplacental antibodies are typically IgG, so would not explain the presence of significant levels of IgA. This may reflect early exposure to rotavirus. A study from a neonatal unit in Vellore reported that approximately 44% of neonates were infected with rotavirus, which could be sufficient to account for the antibody response(125). It is also possible that it could reflect non-specificity of the assay, though it seems unlikely that that would result in a differential result between Vellore and Karonga

Historically, comparing anti-rotavirus IgA titres between populations and between studies has been problematic because of variation in sampling times, assays used and interpretation of results. However over the last decade anti-rotavirus IgA assays have been conducted in a more standardised manner as part of vaccine efficacy and immunogenicity studies, meaning that comparisons of results between populations are now much easier(298). The banks of data describing IgA titres in young infants from different settings resulting from these studies offer a potentially valuable data source to increase our understanding of patterns of exposure in different regions. However, traditional methods of defining seroconversion such as fold increase or the use of cut-offs can be problematic. Fold

increase can under-estimate new infections in those with high levels of pre-existing antibody titre, or over-estimate infections in those with very low level titres at the time of initial sampling(299). The use of cut-offs is also prone to misclassification bias, particularly when the antibody titres of potentially sero-positive or sero-negative individuals overlap. To maintain specificity, cut-offs are often set at a relatively high level, and as a result some truly sero-positive individuals will be misclassified as sero-negative, potentially underestimating the true sero-prevalence of an infection(300).

Mixture models offer potential advantages over these methods as they provide a visually intuitive interpretation of the data, and evaluate the data probabilistically, (i.e. estimate the probability of each sample falling into the positive or negative distribution), thus avoiding the major assumption of an absolute cut off. Boot-strapping can be used to generate confidence limits and estimate uncertainty. With our models, the mean of the two distributions is reasonably constant across models for Vellore and Karonga respectively, increasing confidence that these models would be reproducible and consistent across different datasets.

Improved understanding of patterns of force of infection in LIC could inform evaluations of vaccine effects, and strategies to improve vaccine immunogenicity and performance. In terms of evaluation of vaccine effects, sero-surveys could be used to estimate population level incidence pre and post vaccine introduction in unvaccinated groups. This would contribute to measurement of total vaccine impact, and identification of indirect effects in age groups not eligible for vaccination. In terms of strategies to improve vaccine immunogenicity, if high force of infection leading to high maternal antibodies lowers immunogenicity, an additional dose of vaccine or delayed vaccine schedule may improve vaccine immune response(290). A recent randomised controlled trial (RCT) in Ghana compared the immunogenicity of three doses of RV1 at 6, 10 and 14 weeks to two doses at either 6 and 10 or 10 and 14 weeks, and found that a significantly greater proportion of infants seroconverted (IgA titres≥20 IU) in the three dose group compared to two doses at 6 and 10 weeks(310). In terms of improving vaccine performance, identifying settings such as Vellore with a high burden of very early disease could identify populations where a neonatal dose of vaccine may improve overall vaccine effectiveness. A clinical trial of two doses of RotaShield in Ghana, the first dose given in the neonatal period, demonstrated encouraging vaccine efficacy against rotavirus disease of all severity of 63.1%(311). In addition, the candidate vaccine RV3-BB, currently undergoing immunogenicity trials, is based on a neonatal strain and incorporates a neonatal dose(131).

3.4.1 Limitations

Serum IgA is probably the best marker of recent rotavirus infection currently available, but it is not a perfect correlate of protection(66). While IgA has been shown to increase in response to rotavirus

infection and to correlate with protection against severe disease in several settings(63,64), there is some evidence that it is a less good correlate of vaccine take in low income settings(96), and it has not been possible to define an absolute cut-off to define protection against rotavirus disease(64). In addition, using serology alone to estimate rotavirus incidence will undoubtedly provide an under-estimate, as it is known from cohort studies in infants that around one quarter of infections in children occur without a corresponding rise in IgA titre(38,62). The overall patterns of infection across populations should be comparable, but given that IgA response to rotavirus vaccine is reduced in LIC compared to HIC(298), and that complete protection against severe rotavirus disease seems to require more repeated episodes of infection in some LIC compared to HIC(38), there may be population level differences in immune response to natural rotavirus infection that are not yet fully understood. Furthermore, given the limited evidence derived from this study when comparing IgA responses to symptomatic vs asymptomatic infection, it appears that a substantial proportion of those with asymptomatic infection may not seroconvert. It is therefore possible that use of sero-response could underestimate the rate of asymptomatic infection.

It is also possible that sero-response only captures first infection; thus subsequent infections may not boost IgA levels sufficiently beyond baseline for re-infection to be identified. We however observed a rapid decay in IgA titres (~10 fold per year) following an initial infection, which should allow detection of some episodes of reinfection, particularly those with a wider time interval between infections. This is consistent with findings described by Bernstein et al, where IgA titres in serum declined by 30% within a month of infection, and to a small fraction of their maximal response by a year(312). There are also some logistical challenges to the use of serological data, in that multiple serum samples from young children are required. While there are several existing datasets which could be utilised to explore force of infection using IgA titres prior to vaccine introduction, prospective studies on vaccine impact would likely require large scale sero-surveys which can be difficult to implement, and particularly difficult to implement in LIC where there can be social and cultural barriers to blood collection.

This analysis was based on existing data, and included a small number of children, particularly from Karonga, and it is likely that this contributes to wide confidence bounds around prevalence and incidence estimates, although the differences in exposure patterns remain striking. We do not possess any data on symptoms or viral shedding in stool from the Karonga cohort, should we have wished to investigate the link between IgA response and clinical disease more closely. In addition the timing of collection of the first serum sample differed by site (6 weeks in Karonga vs birth in Vellore). This could be important given the frequency of neonatal infection in some populations(125) and the variation in timings could potentially underestimate the difference in proportion of children with detectable IgA

at the time of first sample in each setting, and potentially influence incidence estimates. A further difference between the two cohorts is the prevalence of HIV exposure in Karonga, which was 28% of infants. Data on HIV exposure in Vellore were not available, but is likely to be much lower. However, as HIV infected infants have comparable IgA responses to those of HIV uninfected infants following rotavirus vaccine and rotavirus does not appear to be more frequent in HIV infected children(260,313,314), it seems unlikely that HIV exposure status should substantially influence IgA responses to natural rotavirus infection.

3.4.2 Implications, conclusions and further work

Whilst this study does not definitively answer any questions, it utilises existing data to act as a "proof of concept", and to offer an improvement on existing traditional methods to interpret rotavirus serology data in order to define incidence. In doing so, it highlights the remarkable heterogeneities in rotavirus transmission which can occur in two different populations, even with relatively similar socioeconomic situations.

This work identifies several areas for further study. Detailed examination of host factors in Vellore and Karonga may offer some insight into the discrepancies in force of infection, particularly if this is at least partly related to immune response in infants. More data on climate and any effect of climate on force of infection in the two populations would also be useful. In addition it would be helpful to understand the relationship between asymptomatic infection and IgA response in more detail, as this would contribute to understanding the role of asymptomatic infection in maintaining protection against rotavirus, and the ability of sero-surveys to account for asymptomatic infection in estimations of incidence.

Unfortunately data on globally licensed rotavirus vaccine performance in these two specific populations are lacking; vaccine trials in Vellore have tended to focus on locally developed or novel candidate vaccines(130), and the vaccine trials in Malawi were conducted in Blanytre, an urban setting with very different population characteristics compared to rural Karonga. Future studies could develop our understanding of the interplay between force of infection and vaccine performance by relating pre-vaccine incidence data to vaccine efficacy and effectiveness at a local level. This should be possible in some sites where vaccine trials have been embedded within on-going surveillance systems, particularly if historic data could be used. For the most part, these data are only available for infants in the first 6 months of life, but it would still offer an opportunity to compare incidence rates and vaccine efficacy between locations. If force of infection plays a role in reduced vaccine effectiveness, it is possible that effectiveness will improve over time, as force of infection declines with vaccine introduction. The methods outlined in this study then could potentially provide a

mechanism to monitor changes in population incidence and relate this to vaccine performance, though this would need to be planned at or near to the time of vaccine implementation.

Understanding rotavirus transmission patterns in different populations is extremely important in both understanding disparities in vaccine performance in different settings, and in considering strategies to address this. Accurate estimates of population level incidence are challenging to obtain, because a large proportion of rotavirus infection and disease occurs at the community level, and the intensive studies required to delineate these are costly, logistically challenging, and place a considerable burden on families. In light of this, using sero-prevalence to estimate incidence, particularly when existing data can be utilised, is potentially both efficient and cost effective. The mixture models developed and applied in this study may offer an improvement on standard methods of analysing serological data in order to define incidence.

Chapter 4. Direct and indirect effects of rotavirus vaccination on rotavirus hospitalisations among children in Malawi four years after programmatic introduction

4.1 Introduction

There is now good evidence of rotavirus vaccine effectiveness and impact from high and middle income countries, which demonstrate vaccine effectiveness similar to pre-licensure clinical trial efficacy (>85%)(315). Early data on vaccine effectiveness from LIC are encouraging compared to efficacy estimates, with vaccine effectiveness estimates ranging from 56% to 75%(186–188), but are sub-optimal compared to high income settings. In this context characterising the ongoing burden of disease and evaluation of the overall population level impact of vaccination programmes in LICs is essential. The previous chapter explored techniques for estimating population level incidence, which is important for understanding discrepancies in vaccine performance between populations, evaluating vaccine impact, and informing interventions. This chapter will focus on describing the residual burden of rotavirus disease in a low-income, Malawian population 4 years after vaccine introduction, and investigating for evidence of rotavirus vaccine indirect effects.

Halloran, Longini and Struchiner(212,316,317) describe how vaccine effects can be divided into 4 components or categories based on different combinations of direct and indirect effects, and define study designs which allow the evaluation of each of these. The four components are defined as firstly the direct effect of the vaccine on the vaccinated individual; secondly the indirect effect of programmatic vaccine introduction on unvaccinated members of the community; thirdly the total effect of the vaccine on a vaccinated individual which is a combination of both direct and community level indirect effect; and finally the overall effect of the vaccination programme which is a population level weighted average of indirect effects in unvaccinated individuals and total effects in vaccinated individuals(317) (Fig 4.1), where indirect effects are defined as reduction in disease burden due to changes in transmission resulting from vaccination.

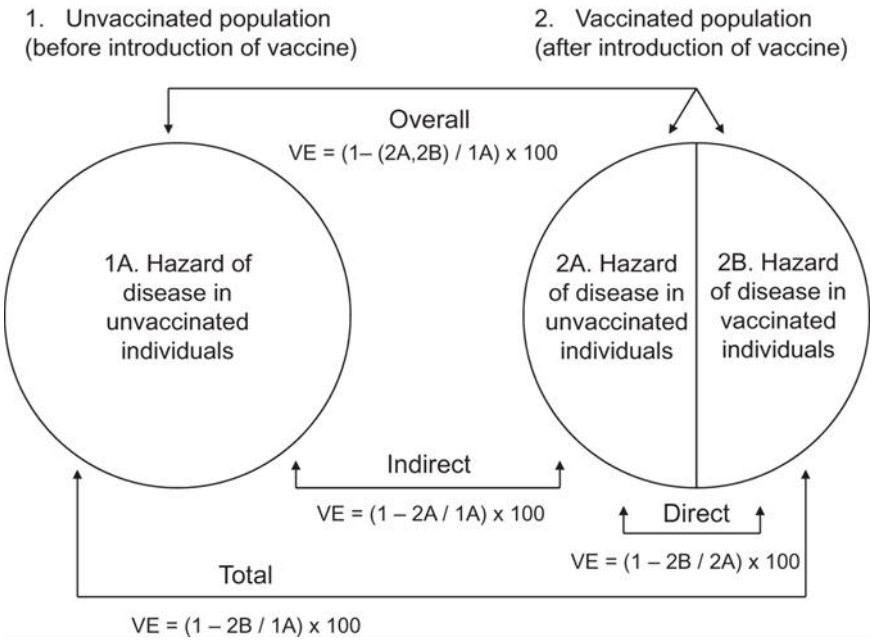


Figure 4.1 Types of vaccine effectiveness as described by Halloran et al(212). A vaccinated population will still have some individuals within the population who are unvaccinated because 100% vaccination coverage is generally never achieved. VE, vaccine effectiveness. Reproduced from **Panozzo et al. Direct, Indirect, Total, and Overall Effectiveness of the Rotavirus Vaccines for the Prevention of Gastroenteritis Hospitalizations in Privately Insured US Children, 2007–2010. Am J Epidemiol. 2014;179(7):895-909**(226)**, by permission of Oxford University Press.**

Evaluation of rotavirus vaccine indirect effects is important, as infants are often reservoirs of infection within a community, and crucial for propagating transmission to other vulnerable members of a population. Infant vaccination programmes against other infectious diseases such as that caused by *Haemophilus influenzae* and *Streptococcus pneumoniae*(318,319) have generated substantial indirect effects which have had a significant, and sometimes unexpected, impact on the overall burden of disease in a population. Incorporating indirect effects of vaccination into cost effectiveness models and considering them when evaluating overall vaccine impact can tip the balance in terms of cost-effectiveness, and can help determine whether vaccines are effective enough to reduce transmission and eliminate disease. In a modelling study of the cost effectiveness of rotavirus vaccine in European settings, it was predicted that 59% of cost savings would be attributable to indirect effects of rotavirus vaccine(320), and a post-implementation cost-effectiveness study in Australia found that the national rotavirus vaccination programme was likely to be cost-saving, at least in part due to unanticipated indirect effects to unvaccinated individuals(229). This is a particularly crucial issue in LIC, as

although GAVI Alliance support has allowed many LIC to successfully introduce rotavirus vaccination, in the long-term immunisation programmes must be locally funded. Using an example from a LIC for a different enteric pathogen, consideration of indirect effects in the evaluation of cholera vaccine led to a conclusion that programmatic cholera vaccination was cost effective, and also confirmed vaccination as a valuable public health measure in the reduction of the cholera burden in endemic settings(246,247).

In view of this, consideration of vaccine indirect effects is gaining increasing prominence as an important component of evaluation of overall vaccine impact(321). Ideally, such studies would be planned and implemented as part of clinical trials prior to vaccine introduction, for example cluster randomised trials which compare risk of disease between unvaccinated individuals in vaccinated clusters and unvaccinated individuals in control clusters unexposed to vaccine(322), or stepped-wedge designs where vaccine is sequentially introduced into different regions with the as-yet unvaccinated region acting as a control arm(323). Randomised prospective studies can minimise the bias and confounding which is challenging to control for in observational studies, and allow evaluation of separate components of vaccine effects through comparisons between different groups within and across clusters(212) (Fig 7.1). However such studies are expensive and logistically challenging to conduct, and for already licensed vaccines with a documented public health benefit such as rotavirus, difficult to justify both ethically and financially. For the most part, we must therefore rely on observational studies to evaluate rotavirus vaccine indirect effects.

The majority of studies which have described rotavirus vaccine indirect effects to date have used surveillance or ecological level data to compare the frequency of rotavirus in unvaccinated groups following vaccine introduction with that observed at baseline (prior to vaccine introduction)(234,235). In addition some studies have estimated the expected reduction in rotavirus infection or disease based on vaccine trial efficacy and vaccine coverage and compared this to observed declines following vaccine implementation, with any additional reduction assumed to be attributable to the indirect effect of the vaccine(219). One study compared the risk of rotavirus disease in household contacts of vaccinated children with the risk of disease in household contacts of unvaccinated children(225), and there are a small number of prospective studies specifically designed to evaluate indirect effects in individual groups(224,226).

There is increasing evidence of rotavirus vaccine indirect effects in infants, children and adults from a variety of high income settings including Europe, Australia, and the USA. Data from middle income countries are also beginning to emerge with evidence of greater than expected reductions in rotavirus disease burden, or reductions in unvaccinated groups described in studies from central Europe, Latin America and Thailand, although intriguingly no evidence of a significant indirect effect was observed in Ghana or South Africa(185,201), middle income countries in sub-Saharan Africa. Currently the only data on rotavirus indirect effects from a LIC is Rwanda, where reductions in rotavirus hospitalisations were seen in all children under 5 years, including those age groups not age-eligible for vaccination(202). These findings are summarised in detail in Chapter 1, section 1.6.2, page 63.

Obtaining direct data from low income settings is crucial both because these are the settings where indirect effects could make a substantial contribution to overall vaccine effects and therefore the economic viability of long term vaccination programmes, and because differences in the population between low and high income settings mean that data cannot be extrapolated from one to the other. LICs differ from HIC and MIC in terms of population structures and density, water and sanitation provision, and prevalence of underlying co-morbidities such as malnutrition and HIV. These factors may influence the incidence and transmission epidemiology of rotavirus infection and disease, and may mean that indirect protection differs from that documented from HIC(215).

This study aimed to describe rotavirus epidemiology and investigate the presence and extent of rotavirus vaccine indirect effects 4 years after programmatic vaccine introduction in Blantyre, Malawi utilising a cohort of children recruited into an existing surveillance platform. Firstly, the overall change in prevalence of rotavirus in all children hospitalised with AGE since vaccine introduction, regardless of vaccine status, was described. Direct VE estimates were then updated using a case-control design nested within the surveillance platform. Following this rotavirus prevalence in unvaccinated children pre- and post- vaccine introduction was compared, and finally the observed reduction in incidence of rotavirus hospitalisation was compared to that expected based on vaccine coverage and trial efficacy estimates to obtain an estimated magnitude of rotavirus vaccine indirect effects in this setting.

4.2 Methods

4.2.1 Objectives

1. Describe change in rotavirus prevalence in admitted children with AGE since vaccine introduction

2. Estimate age stratified vaccine effectiveness for rotavirus gastroenteritis

3. Describe change in rotavirus prevalence in unvaccinated children with AGE since vaccine introduction

4. Compare observed with expected reductions in admitted rotavirus AGE incidence following vaccine introduction

4.2.2 Study design

Data for this study arose from a prospective diarrhoeal surveillance platform with a nested case control study for evaluation of rotavirus vaccine effectiveness.

4.2.3 Study site

This study was conducted at QECH, Blantyre, Malawi. Surveillance for rotavirus gastroenteritis has been conducted at QECH since 1997, but enhanced surveillance was commenced in January 2012 in advance of planned national introduction of rotavirus vaccine. This analysis includes data from this period until the end of June 2016.

4.2.4 Study population

This study recruited rotavirus vaccine age eligible children presenting to QECH with a diagnosis of acute gastroenteritis, defined as ≥3 loose stools in a 24 time period.

4.2.5 Study procedures

The surveillance platform has been described in detail in the literature(116,186). Children who present to QECH with acute gastroenteritis (AGE) during routine clinical hours were identified by research nurses, and enrolled following informed consent. Surveillance included children admitted to the main paediatric ward (special care), the nursery (children under 6 months of age), and the malnutrition unit, as well as children treated as outpatients in the accident and emergency department. Following enrolment, detailed demographic and clinical data were recorded, anthropometric assessment undertaken and a bulk stool sample collected. HIV testing was conducted according to Ministry of

Health national guidance(278). Vaccine status was obtained from government-issued family-held records (Health Passport). Disease severity was defined using the 20-point Vesikari score(324), where a score of ≥11 indicates severe disease. HIV infection was defined based on a positive rapid test (over 12 months of age), or positive HIV DNA PCR (under 12 months of age). HIV exposure was defined as a positive maternal HIV rapid test. Nutritional status was assessed using WHO standards, where severe acute malnutrition (SAM) was defined as any one of weight for height Z score (WHZ)<-3 SD from WHO standard, mid-upper-arm circumference (MUAC)<115mm, or nutritional oedema(325).

4.2.6 Laboratory methods.

Stool samples were processed on arrival in the laboratory, and stored as 10% suspension in PBS at 4-7 degrees Celsius before batch testing weekly for rotavirus antigen using enzyme immunoassay (EIA, Rotaclone, Meridian Bioscience, Cincinnati, Ohio). Following testing samples were stored at -80^{o}C . HIV testing of mothers and children was conducted using the government programme of two sequential EIA rapid tests (Determine HIV-1/2 [Abbott Laboratories, USA] and Uni-Gold HIV [Trinity Biotech PLC, Ireland]), or HIV DNA PCR for infants under one year of age(278).

4.2.7 Statistical analysis

4.2.7.1 Descriptive analysis

Continuous variables were summarised using mean and standard deviation (SD) for normally distributed data, or median and interquartile range otherwise. Differences in independent categorical covariates were assessed using Chi squared tests. Student's t or rank sum tests were used to compare independent means or medians respectively. Vaccine coverage was described using those children with AGE who tested negative for rotavirus.

4.2.7.2 Prevalence changes over time of EIA positive rotavirus

As QECH is the only government facility which admits children in the district GE admissions for rotavirus were analysed over time as a prospective cohort. Poisson regression models were used with robust standard errors(326) to evaluate year-on-year differences in rotavirus prevalence in hospitalised gastroenteritis, with the year preceding introduction as baseline. Relative risk (RR) was preferred to odds ratios (OR) due to the tendency of OR to overestimate effect size when outcomes are not rare. Variables evaluated as potential confounders were age, household size, month of admission, HIV infection, HIV exposure

and presence of severe acute malnutrition. Univariate analysis of potential confounding variables was initially conducted by constructing two by two tables and using chi-squared tests for categorical variables, and Mantzel-Haensel odds ratios for continuous variables. Potential confounders were then tested in the Poisson model, with comparison between models conducted using Akaike Information Criterion (AIC). No significant confounders of the relationship between time since vaccine and RR of rotavirus positive gastroenteritis were identified, however models were adjusted empirically for age and month of presentation, and analysis restricted to the first 6 months (January to June) of each year for consistency.

4.2.7.3 Time series analysis

Time series analysis was used to describe trend and seasonality in the prevalence of rotavirus in hospitalised diarrhoeal cases over time. Mean monthly proportion of rotavirus in stools was defined by collapsing the dataset against number of diarrhoeal stools sent per month and number of rotavirus positive cases per month. A 5-month locally weighted smoother (defined as (1/8)*[1*x(t-2) + 2*x(t-1) + 2*x(t) + 2*x(t+1) + 1*x(t+2)]) was applied to this value to define seasonality and a 13-month locally weighted smoother (defined as (1/24)*[1*x(t-6) + 2*x(t-5) + 2*x(t-4) + 2*x(t-3) + 2*x(t-2) + 2*x(t-1) + 2*x(t) + 2*x(t+1) + 2*x(t+2) + 2*x(t+3) +2*x(t+4) + 2*x(t+5) +1*x(t+6)], where x(t)= percentage rotavirus-positive stools per month) was applied to the same to define secular trend. A linear model was then used to assess trend in rotavirus prevalence over time.

4.2.7.4 Vaccine effectiveness

Using a nested case-control study design, unconditional logistic regression was used to estimate percentage vaccine effectiveness (VE) using (1-OR*100) for rotavirus vaccine (using 2 doses of vaccine vs 0 doses) among rotavirus positive gastroenteritis cases vs test-negative gastroenteritis controls. VE was adjusted for age, and for secular and seasonal fluctuations using year and month of admission, consistent with previous VE estimates from our group. Only children with documented vaccine status who were age eligible for both doses of vaccine (i.e. who were born on or after the 29th September 2012) were included. As 15% of children did not have hand held health passports, a sensitivity analysis was performed on children with undocumented vaccine status.

4.2.7.5 Estimating indirect vaccine effects

Firstly, Poisson regression models with robust standard errors were used to evaluate any change in rotavirus prevalence in unvaccinated admitted infants (<12 months of age) and children (12-59 months of age) with gastroenteritis pre and post vaccine introduction. All available data were included in this. Secondly the observed reduction in incidence of hospitalised rotavirus gastroenteritis was calculated year by year following programmatic vaccine introduction, and compared to the estimated expected reduction in incidence (expected direct effect), making the assumption that any additional reduction in observed incidence was due to rotavirus vaccine indirect effects(240). As above, because enhanced surveillance data were not available for a full calendar year prior to rotavirus vaccine introduction, incidence estimates were calculated for the first 6 months of each year to minimise risk of any seasonal bias.

For this analysis incidence was defined as case numbers of admitted rotavirus gastroenteritis per 100,000 age stratified population per 6 month time interval using projected population estimates for Blantyre city from the National Statistics Office of Malawi. Data on the proportion of infants were not available for Blantyre city, so this was assumed to be equivalent to that reported at a national level. The expected direct effect was calculated by multiplying vaccine coverage by vaccine efficacy, using a value of vaccine efficacy of 49.4% for severe disease in infants, 34.5% for all rotavirus gastroenteritis in infants and 17.6% for children with severe disease, and where both vaccine coverage and efficacy were expressed as proportions(146,327). There were no efficacy estimates published for disease of all severity in children so expected effects were not calculated for this group.

The observed effect was calculated by comparing annual post-vaccination incidence from January to June to incidence for January to June 2012 (prior to vaccine introduction) using:

*(Pre-vaccine incidence – post-vaccine incidence)/pre-vaccine incidence*100.*

The indirect effect was then estimated by subtracting the calculated expected effect from the observed effect(240).

To investigate for secular trends in diarrhoeal admissions to QECH, the incidence rate of hospitalised test-negative gastroenteritis was estimated. The denominators above were used to estimate child years at risk/100,000 population by dividing the total number of days at risk by 365, then multiplying this by the population denominator /100,000. For the

pre-vaccine period the population estimate for 2012 was used, and for the post vaccine period the estimated population at the post-vaccination midpoint was used (September 2014).

Throughout, analyses were restricted to children with stool samples collected. With the exception of VE estimates, all analyses were restricted to admitted children. For assessment of vaccine indirect effects children up to 59 months of age were included because of the potential impact of indirect effects in this group, however for the remainder of the analysis children were categorised into <12 month or 12-23 month age groups.

4.2.8 Ethics Ethical approval was obtained from the National Health Sciences Research Committee, Lilongwe, Malawi (867), and by the University of Liverpool Research Ethics Committee (000490).

4.3 Results

Stool samples were collected from 2320 children (median age 10.68 months, interquartile range [IQR] 7.72, 15.29) between 1st January 2012 and 30 June 2016. 1318 infants were eligible for both doses of rotavirus vaccine, and 1130 had documented evidence of vaccine status. Characteristics of the population are shown in Table 4.1. High levels of vaccine coverage were reached within 6 months of vaccine introduction (Fig. 4.2).

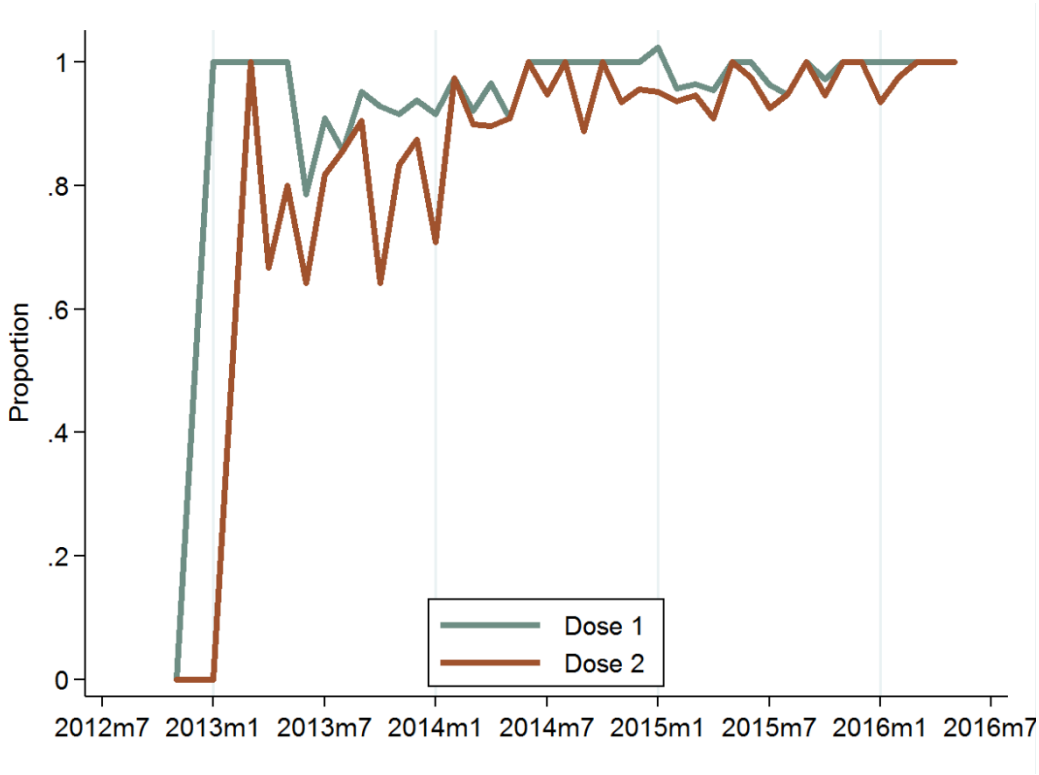


Figure 4.2 RV vaccine coverage in vaccine age eligible rotavirus test negative children presenting to QECH with AGE.

Table 4.1. Characteristics of study population

Characteristic		Denominator
Male (n, %)	1339 (57.77)	2318
Age in months (median and IQR)	10.68 (7.72, 15.29)	2320
Weight for height Z score (WHZ)* (mean and SD)	-0.92 (1.88)	2292
Severe acute malnutrition (n, %)*	413 (18.05)	2288
RV coverage** (n, %)		
0 doses	43 (3.81)	1130
1 dose	60 (5.31)	1130
2 doses	1027 (90.88)	1130
HIV†		
Infected (n, %)	71 (4.03)	1761
Exposed (n, %)	426 (18.80)	2266

*Weight corrected by adding 10% to weights for those with severe disease to account for dehydration **in those vaccine age-eligible with health record confirmation. †HIV infected is defined as a positive HIV rapid test over 12 months of age, or a positive HIV DNA PCR result. HIV exposed is defined as a positive maternal HIV rapid test.

4.3.1 Overall decline in rotavirus prevalence

The relative risk of rotavirus being detected among children admitted to QECH with diarrhoeal disease has consistently declined compared to the pre-vaccine baseline (Table 4.2 and Fig. 4.3A). Despite this, over 25% of all gastroenteritis admissions remain rotavirus positive (Table 4.2). This remained true when yearly time intervals following vaccine introduction were used, with 61/234 (26%) gastroenteritis stools positive for rotavirus from November 2015 to June 2016 (Table A2, Appendix, page 260).

Table 4.2. Relative risk of rotavirus detection in children admitted to QECH with gastroenteritis

	RV** negative	RV positive	Total	RR (95% CI[†])*
Time period				
Pre-vaccine (Jan'12- Jun'12)	110 (56.70)	84 (43.30)	194	1 (Ref)
Jan'13- Jun'13	185 (58.18)	133 (41.82)	318	0.95 (0.78 -1.16)
Jan'14- Jun'14	177 (69.96)	76 (30.04)	253	0.77 (0.61 -0.98)
Jan'15- Jun'15	219 (75.26)	72 (24.74)	291	0.60 (0.46-0.77)
Jan '16- Jun'16	132 (72.13)	51 (27.87)	183	0.74 (0.57-0.98)
Total	823 (66.42)	416 (33.58)	1239	

*adjusted for age in months and month at admission. Relative risk for rotavirus gastroenteritis vs test-negative gastroenteritis [†]95% confidence interval **Rotavirus (RV)

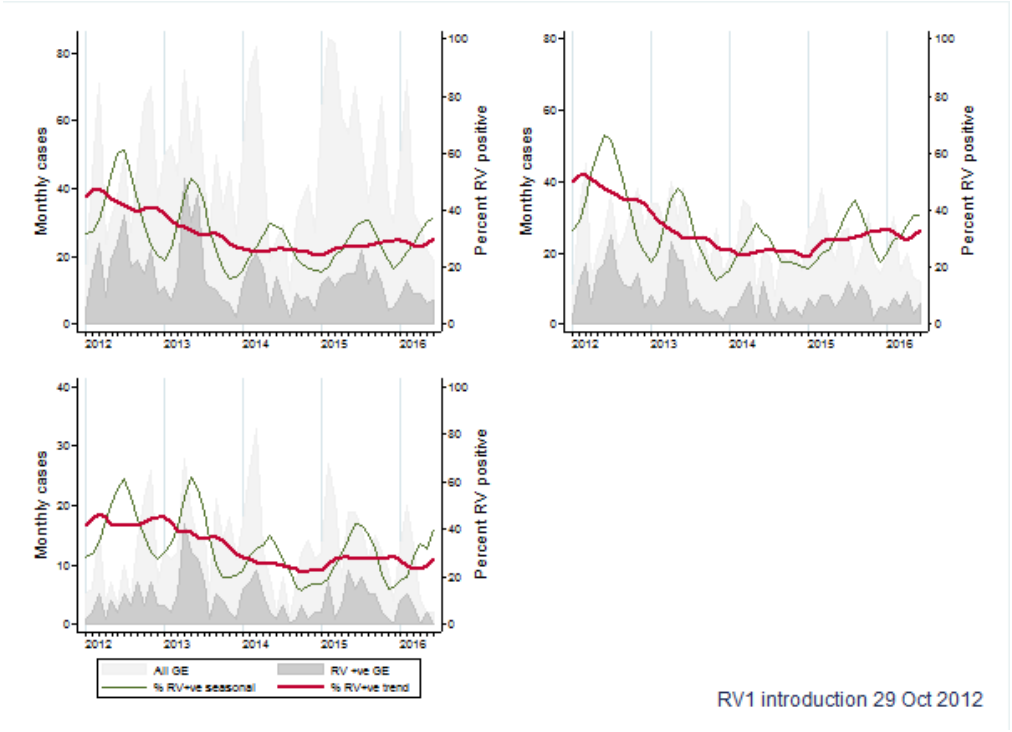
Following vaccine introduction, the median age of cases has increased significantly from 9.48 months (IQR 7.00, 13.54) prior to vaccine introduction, to 10.86 months (IQR 7.95, 15.41) months (rank sum test p<0.001). The adjusted relative risk of rotavirus positivity among infants hospitalised with gastroenteritis in the first 6 months of the year has decreased from 69/139 [49.64%] to 197/607 [32.45%] since vaccine introduction (adjusted RR 0.67 [95% CI 0.55, 0.82] p<0.001).

This effect is smaller in children aged 12-23 months, where the relative risk pre- and post-introduction respectively was 15/37 (40.54%) and 122/352 (34.66%) (adjusted RR 0.85 [95% CI 0.57, 1.28] p=0.440) (Fig. 4.3A). The proportion of admitted rotavirus positive cases aged 12-23 months increased from 15/84 (17.86%) in the January to June period prior to vaccine introduction to 122/319 (38.24%) in the same months subsequent to vaccine introduction (chi squared p<0.001).

Five month and 13 month smoothers provided a good fit to the seasonal and secular trends in proportion of rotavirus positive stools over time (Fig. 4.3B). Clear seasonality in rotavirus prevalence is demonstrated, with some blunting of this seasonal picture following vaccine introduction (Fig. 4.3A&B). Linear regression showed a significant negative trend in prevalence of rotavirus over time in infants (regression coefficient -0.36 [95% CI -0.46, -0.25] P<0.001) and in children aged 12 to 23 months of age (regression

coefficient -0.43 [95% CI -0.51, -0.36] p<0.001), where the regression coefficient represents the percentage change in rotavirus positivity per month.

A



B.

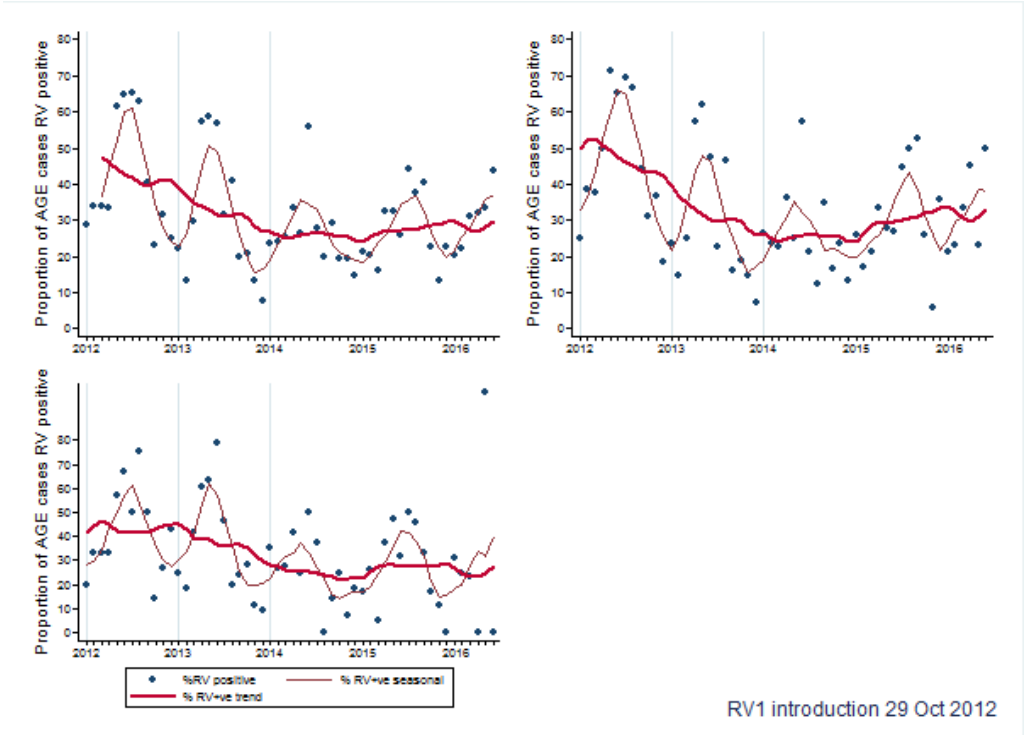


Figure 4.3 A Monthly diarrhoeal and rotavirus positive admissions to QECH over time, with secular and seasonal trends in rotavirus prevalence B. Fit of smoother to raw data for monthly rotavirus prevalence

4.3.2 Vaccine effectiveness estimates

The adjusted VE for two doses of vaccine, across all ages and disease severity was 61.89% (95% CI 28.04%, 79.82%) (Table.4.3). VE against severe disease among children <12 months of age was 85.94% (95% CI 58.64%, 95.22%). VE against severe disease in the 12-23 month age group was 20.40% (95% CI -412.74%, 87.64%). Results were similar when analysis was limited to passport confirmed vaccine status or when undocumented vaccine status was included. Reported results are for passport confirmed vaccine status.

Table 4.3. Vaccine effectiveness estimates

	N (Rotavirus positive 2 dose RV1 [%])**	(N Rotavirus positive 0 dose RV1 [%])**	Vaccine Effectiveness (95% CI)	P value
Adjusted*				
2 doses	275/1019 (26.99)	20/43 (46.51)	61.89 (28.04-79.82)	0.003
Disease severity* (all ages)				
Severe	237/754 (31.43)	15/23 (65.22)	78.95 (48.25-91.44)	0.001
Mild/mod	36/249 (14.46)	4/19 (21.05)	14.20 (-193.39-74.90)	0.807
By age* (all severity)				
<12 m	190/696 (27.30)	16/29 (55.17)	74.88 (44.59 -88.61)	0.001
12-23m	78/285 (27.37)	4/13 (30.77)	31.69 (-139.03-80.48)	0.551
By age* (severe disease)				
<12m	160/521 (30.71)	13/18 (72.22)	85.94 (58.64-95.22)	<0.001
12-23	71/208 (34.13)	2/5 (40.00)	20.40 (-412.74-87.64)	0.810

*Adjusted for age, and year and month of presentation. All are two-dose estimates. **Number of rotavirus positive cases; the denominator is all gastroenteritis cases with stool sample collected. RV1=monovalent rotavirus vaccine

4.3.3 Indirect vaccine effects

Among unvaccinated infants with gastroenteritis, rotavirus prevalence declined from 117/221 (52.94%) in the 10 months prior to vaccine introduction to 65/184 (35.33%) in the 14 months following vaccine introduction, (adjusted RR 0.70 [95% CI 0.55, 0.88] p=0.003) (Fig 4.4A). Linear regression showed a significant negative trend in prevalence of rotavirus over time (regression coefficient -0.73 [95% CI -0.86, -0.60] P<0.001). In unvaccinated children 12-59 months of age with severe disease, there was no evidence of a decline in the prevalence of rotavirus following vaccine introduction, with 26/84

(30.95%) rotavirus positive pre-vaccine introduction and 70/193 (36.27%) rotavirus positive after introduction, RR 1.14 (95% CI 0.79, 1.63), and no evidence of a significant decline on linear regression of trend (regression co-efficient 0.07, 95% confidence interval -0.22 to 0.36, p=0.634). This analysis was truncated at 24 months from the start of surveillance because the vast majority of infants after this time point were vaccinated. This truncation limited the ability to describe rotavirus seasonality in unvaccinated children pre- and post-vaccine introduction, but the 5 month (seasonal) and 13 month (secular) smoothers provided a good fit to the data (Fig 4.4 B)

Comparing the observed against expected reduction in incidence showed a difference of between 9 and 24% in admitted infants with rotavirus gastroenteritis of any severity (Table 4.4). Restricting to infants with severe rotavirus gastroenteritis, no difference was seen. There was also no evidence of an indirect effect demonstrated on comparison of observed vs expected reductions in incidence in children aged 12-59 months (Table 4.4)

Table 4.4. Comparison of expected and observed vaccine effects by year since vaccine introduction

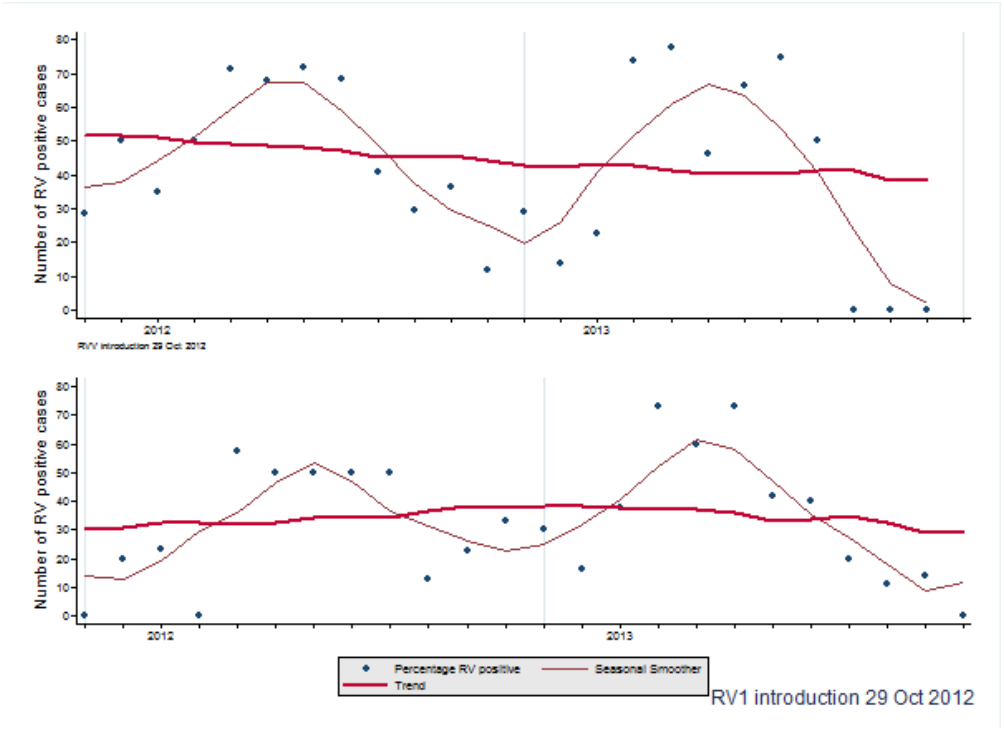
	Incidence*	RV coverage** (%)	Expected effect (%)	Observed effect (%)	Difference in observed effect (%)
<12m severe RV GE					
Jan '12- Jun'12†	149	-	-	-	-
Jan '13- Jun'13	194	29.46	14.56	-30.09	-44.64
Jan '14- Jun'14	104	92.31	45.60	30.28	-15.31
Jan '15- Jun'15	117	94.12	46.49	21.72	-24.77
Jan '16- Jun'16	99	94.29	46.58	33.62	-12.95
All post vaccine	129	77.48	38.11	13.89	-24.23
<12m all RV GE					
Jan '12- Jun'12 †	234	-	-	-	-
Jan '13- Jun'13	256	29.41	10.21	-9.22	-19.43
Jan '14- Jun'14	139	90.08	31.26	40.73	9.47
Jan '15- Jun'15	123	94.32	32.73	47.45	14.73
Jan '16- Jun'16	102	92.31	32.03	56.39	24.13
All post vaccine	149	73.16	25.39	33.84	08.45
12-60 m severe RV GE					
Jan '12- Jun'12 †	13	-	-	-	-
Jan '13- Jun'13 ††	58	-	-	-	-
Jan '14- Jun'14	25	63.23	11.13	-85.91	-97.04
Jan '15- Jun'15	26	90.77	15.96	-94.23	-110.21
Jan '16- Jun'16	14	96.43	16.97	-07.46	-24.43
All post vaccine	22	85.82	15.10	-62.53	-77.64

*6 month Jan to June, per 100,000 infants (<1 year). **% coverage for 2nd dose of rotavirus vaccine †prior to vaccine introduction †† period from Jan '13 to Jun '13 excluded for children over 12months as these children were not vaccine age eligible.

In contrast to the incidence rate of rotavirus-positive hospitalisations, the incidence of hospitalisation for rotavirus-negative gastroenteritis increased over time. The incidence rate of hospitalisation with rotavirus-negative gastroenteritis among infants was 479 per 100,000 child-years at risk prior to rotavirus vaccine introduction and 655 per 100,000 child-years at risk subsequently (Incidence rate ratio 1.37 [95% CI 1.06, 1.79]). For children, the incidence rate for hospitalisation with rotavirus-negative gastroenteritis was

77 per 100,000 child years pre- and 136 per 100,000 child years post-rotavirus-vaccine introduction (Incidence rate ratio 1.77 [95% CI 1.27, 2.52])

A.



B.

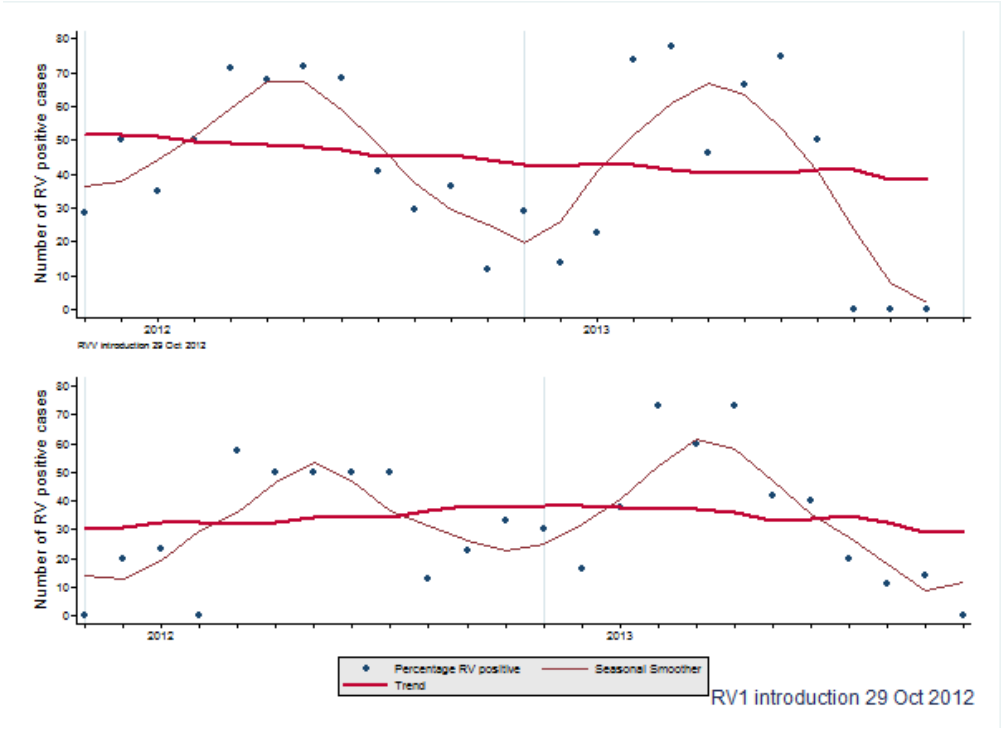


Figure 4.4A Monthly diarrhoeal and rotavirus positive admissions to QECH over time in (a) unvaccinated infants and (b) children 12-59 months, with secular and seasonal trends in rotavirus prevalence. Data truncated at 24 months from start of surveillance (14 months from vaccine introduction) due high vaccine coverage. Fig 4.4B Fit of smoother to raw data for monthly rotavirus prevalence

4.4 Discussion

Four years following programmatic rotavirus vaccine implementation in Malawi, the prevalence of rotavirus in children presenting to hospital with gastroenteritis consistently declined. Vaccine effectiveness against all rotavirus disease was 61.89%, (95% CI 28.04-79.82%). Notably however, a less pronounced reduction in disease and lower VE was observed in children 12-23 months of life compared to those under 12 months of age. There remains a persistent residual burden of disease, with rotavirus responsible for over 1 in 4 gastroenteritis admissions despite high levels of rotavirus vaccine coverage. There was some evidence of a reduction of all hospitalised rotavirus disease in unvaccinated infants, cautiously attributed to indirect vaccine effects, but no evidence of indirect effects were identified in older children or those with severe disease.

In this study high vaccine effectiveness in infants with severe disease was observed (85.94% [95% CI 58.64%, 95.22%] , although this should be interpreted with some caution in view of the large confidence bounds. This finding is consistent with previously reported estimates for Malawi and with estimates from Rwanda(116,187). Our vaccine effectiveness estimates, particularly in children under 12 months of age, have been consistently greater than vaccine efficacy estimates obtained in the same setting from pre-licensure clinical trials, and similar effects have also been seen in other sub-Saharan African countries(187). One explanation for this could be the presence of vaccine indirect effects increasing the measured VE. By design, the case control study for VE should estimate direct VE, as it included only children age-eligible for vaccination and therefore part of a vaccinated population (Fig 4.1). However a modelling study demonstrated the potential for the presence of indirect effects of vaccination to bias measurement of vaccine effectiveness upwards in a situation where vaccine coverage is heterogenous within a population – a situation which is likely in real world post-implementation studies(328).

Despite the encouraging VE estimates in infants and high vaccine coverage, there remains a substantial burden of rotavirus disease in children attending QECH. This is consistent with findings from other LICs(116,185,202,329), and is likely to reflect a combination of sub-optimal vaccine effectiveness and high force of infection. Of interest, however, a residual burden of disease following vaccine introduction has also been noted in some HIC where vaccine effectiveness is high, raising the possibility of population groups other than infants contributing to rotavirus transmission(234). The current study has the major

advantage of being based in a long-standing surveillance system. This will allow ongoing evaluations of trends in rotavirus incidence over time, and will become increasingly important as high vaccine coverage will ultimately prohibit further case-control analysis of VE.

The point estimates of VE in children aged 12 to 23 months are considerably lower than those observed in infants; and although again this should be interpreted in the light of very wide confidence intervals and a significance test which is non-significant at the 5% level, the point estimate still reflects the maximum likelihood value, and is worthy of cautious consideration. This is particularly true given that this finding is corroborated by the relative risk of rotavirus gastroenteritis compared to test-negative controls which has not decreased in this older age group, and the proportion of rotavirus cases occurring in children 12 to 23 months of age which has increased from 21% to 38% following vaccine introduction. Some middle income countries, for example Colombia and Brazil(171,174), have also reported non-significant lower point estimates for VE in the second year of life, but others, including sub-Saharan African countries such as South Africa and Botswana(201,207) have demonstrated protection that is both significant at the 5% level and consistent into the second year of life. Rwanda, the only other LIC which has currently published data on VE in the second year of life, has reported an adjusted VE in the second year of life of 81% (95% CI 25-95)(187).

The observed decline in VE in the second year of life in Malawi could represent a number of phenomena. It could represent evidence of immunological waning, supported by the finding of a large randomised controlled trial in Malawi which showed a higher point estimate of vaccine efficacy in the second year of life among infants given a three-dose RV1 schedule(327), although this study was under-powered to investigate this formally. Alternatively, it could represent an epidemiological effect caused by high background force of infection in LICs, which could lead to an increase in similarity between cases and controls as controls acquire natural immunity through wild-type rotavirus exposure over time, and an artificially reduced VE(145). If this is the case it is possible VE estimates in the second year of life might improve over time as force of infection decreases as a result of vaccination. It may also reflect uncertainty in point estimates or a type two error, resulting from small numbers of children in the older age group. Due to high levels of vaccine coverage in this population it is not possible to recruit further children into the case control study to address this further.

There are currently very little data on rotavirus vaccine indirect effects from LICs(187). This study describes reductions in the relative risk of rotavirus-positive gastroenteritis in unvaccinated infants in the year following vaccine introduction, though this analysis was also limited by high vaccine coverage resulting in a short time period for analysis, and the findings should be interpreted with caution in light of this. Because of the limited data available, data were included for all months of the year for this analysis, acknowledging the possibility that this may introduce some seasonal bias. A sensitivity analysis was conducted restricting to the first 6 months of the year; this resulted in a similar trend to that observed in the full period analysis, although the results were no longer significant at the 5% level. When observed reductions in incidence were compared with those expected, greater reductions were identified in infants with disease of any severity than would be explained by direct effects alone. However this effect was lost when analysis was restricted to those infants with severe disease, possibly reflecting the larger value for vaccine efficacy in this group.

There was also no evidence of an indirect effect of vaccine in older children, contrary to observations from HIC. One potential explanation for this is patterns in age distribution of disease in LIC. Prior to rotavirus vaccine introduction the majority of rotavirus disease in Malawi occurred in the first year of life, with very little rotavirus disease presenting in children over two years of age(260), possibly as a result of high exposure in infancy and subsequent robust immune protection as an older child. On programmatic rotavirus vaccine introduction into such settings older children may therefore be less susceptible to changes in transmission due to a reduction in disease in infants. If true, there may be an increase in identifiable indirect effects in older age groups as force of infection decreases with ongoing vaccination. Detection of any indirect effect in older children could also be made less likely if there is a shift of the burden of disease into the second year of life. In support of this explanation, modelling studies from European settings have predicted that early herd protection could be countered by an later increase burden of disease in children over 12 months of life(330).

These estimates of indirect effects are minimum estimates, as they only capture the effect on hospitalised cases which represent a small proportion of the community burden of rotavirus disease. Additionally if the observed increase in incidence of hospitalisation with rotavirus-negative diarrhoeal disease following vaccine introduction reflects secular trend towards increased presentation and admission with AGE, this could bias estimation of vaccine impact based on hospitalised cases, and estimation of indirect effects derived

from this, towards the null. It should also be acknowledged that these methods of estimating indirect effects are very sensitive to the assumptions used in the calculations. I have used trial vaccine efficacy to estimate the predicted reduction in burden of rotavirus disease because vaccine effectiveness estimates from case control studies may be biased in the event of uneven vaccine coverage(328), however the vaccine efficacy estimates are smaller than the vaccine effectiveness estimates which may bias towards detection of indirect effects. Additionally vaccine coverage was calculated as a monthly average from children presenting to QECH, which may not be representative of coverage throughout the community, although coverage estimated from QECH is consistent with the national level coverage reported by the DHS (91% for two doses in the 2015-2016 DHS(331). Inaccuracies in vaccine coverage could also bias estimates of indirect effects.

The reason for the observed increase in incidence of test-negative diarrhoeal cases observed following vaccine introduction is unclear, but could represent secular changes in health-care seeking behaviour or admission patterns with time. For example, trends in improvement in child health in Malawi and increasing maternal education around health care seeking could result in increased presentations with diarrhoeal disease, and a lower threshold for admission if children do attend. It also may reflect under-ascertainment or unusually low frequency of presentation or admission in the year prior to vaccine introduction. Unfortunately the relatively short period of enhanced surveillance before this point limits assessment of trends in rotavirus prevalence prior to vaccine introduction. Secular changes in denominators can affect prevalence estimates, and as such ongoing surveillance is required to evaluate the long term impact of vaccine in this setting.

4.4.1 Implications, conclusions and further work

In this study a consistent decline in the prevalence of rotavirus in hospitalised GE cases was documented following rotavirus vaccine introduction, and alongside encouraging VE estimates in young infants, particularly those with severe disease. However a residual burden of rotavirus disease was demonstrated, with rotavirus remaining responsible for over a quarter of all AGE admissions, and an apparent reduced vaccine effectiveness in the second year of life. Evidence of an indirect effect of rotavirus vaccine in infants with hospitalised disease was noted, but this was not seen in those with more severe disease or children over 12 months of life. Further observational evaluations of vaccine effectiveness or indirect effects in unvaccinated groups in this setting are challenging as

vaccine coverage is now so high that identifying unvaccinated children is very difficult, and any children who remain unvaccinated are likely to be subject to selection bias.

Several of the phenomena identified in this study, such as apparent reduced vaccine effectiveness in the second year of life and the lack of indirect effect in children over 12 months, may be a result of high force of infection in Malawi, and it is possible that as force of infection and ongoing transmission declines with long term wide spread vaccination programmes some of these effects will change. To monitor this and evaluate ongoing changes in prevalence and incidence, continued surveillance is necessary. In order to gain a better understanding of the trends and to investigate the presence and extent of such effects in greater depth more complex mathematical modelling may be required, particularly in light of the limitations of observational studies in the context of high vaccine coverage

Although tremendous progress has been made over the past decade in reducing the global burden of rotavirus disease and protecting children from rotavirus attributable morbidity and mortality, rotavirus remains a significant cause of AGE in Malawi. If the persistent burden of rotavirus disease continues, interventional studies may be indicated to determine methods to improve vaccine effectiveness or reduce immunological waning in the second year of life. These have been outlined in previous chapters (Chapter 3, section 3.4, page 120) but could include changes or additions to the vaccine schedule such as an additional or delayed dose of vaccine, or novel candidate vaccines. To decide if such strategies are required, and to inform public health strategy to best protect young infants from rotavirus disease, a greater understanding is needed of the overall population level impact of rotavirus vaccines in the poorest countries, including indirect effects, and direct vaccine effectiveness in sub-groups, and how these evolve over time.

RESULTS

SECTION B

Chapter 5. Household transmission of rotavirus in Blantyre, Malawi

5.1 Introduction

The two chapters in Section A have outlined the importance of understanding population level rotavirus transmission in LIC in the context of reduced vaccine effectiveness and a persistent burden of rotavirus disease, and discussed the potential for rotavirus vaccine to reduce transmission through an overall reduction in the force of rotavirus infection. In addition to this, rotavirus vaccine has the potential to reduce transmission by reducing the infectiousness of an index case, or by horizontal transmission of vaccine virus shed in the stool of a vaccinated infant to their household contacts(215). Understanding these phenomenon requires study of the transmission of wild-type and vaccine-type rotavirus between individuals, and Section B will focus on this. The current chapter will focus on defining transmission rates for rotavirus to household contacts exposed to a rotavirus positive index case. Subsequent chapters will explore the relationship between viral shedding density and symptoms, investigate risk factors for rotavirus transmission and investigate horizontal transmission of vaccine type virus within a household.

There is reasonable evidence that rotavirus vaccine has the potential to reduce the infectiousness of an index case. Rotavirus vaccination aims to mimic natural rotavirus infection, which is known to protect against severe rotavirus disease to a degree which varies by location(38,62). Studies from India and South America have shown that the severity of rotavirus diarrhoea correlates with the quantity of virus shed in the stool and that the presence of symptoms is related to the risk of transmission(106,255). Rotavirus vaccination may therefore both diminish viral shedding following exposure to natural rotavirus infection and reduce secondary transmission rates in households, even in the event of clinical vaccine failure. Such effects have been demonstrated with other vaccines such as pertussis(332), and, given the high disease burden of rotavirus, have the potential to contribute substantially to the reduction in the burden of disease in the community, and the overall cost effectiveness of the vaccine programme.

The first step in understanding what may reduce rotavirus transmission is an accurate description of rotavirus secondary attack rate (SAR). SAR is defined as the number of new cases among contacts of an index case(248). Describing SAR is important in itself, as it allows comparisons of transmission rates between populations, and within populations before and after interventions. Persons infected with rotavirus can have symptoms of

gastroenteritis (rotavirus disease), or can have detectable rotavirus shedding in their stool but remain asymptomatic (rotavirus infection). SAR needs to be estimated separately for each of these, as the effect of interventions may differ between them. In addition to this, SAR also forms the basis of estimating important parameters such as the basic reproductive number (R0), which is the number of secondary cases generated by one infectious case in a fully susceptible population(212). Accurate estimates of R0 are crucial in infectious disease epidemiology as an R0 below 1 indicates that it is possible for transmission to be interrupted. However directly measuring R0 for rotavirus is not possible because the ubiquitous nature of rotavirus and the fact that it generates partial immunity make it extremely difficult to identify a fully susceptible population.

Households studies are a well-established method of investigating attack rates for infectious diseases(333–337), and provide appropriate sampling frames to measure rotavirus SAR as they contain a defined group of individuals within close physical proximity to an infectious rotavirus case. Although other small communities have also been used to describe rotavirus SAR, including nurseries and closed communities such as kibbutz(261,338,339), households are relatively practical and efficient to recruit, which is particularly important for studies conducted in low income settings(322). As outlined in chapter 1, the majority of data on rotavirus transmission comes from unvaccinated populations in high income settings including Europe, the US and Australasia, where SARs have been reported to range widely from 12-91% for infection, and 26-86% for disease, depending on the age of the contact and the study design used(76,251,266). All studies employed EIA or EM to diagnose rotavirus which have low sensitivity for the low levels of viral shedding associated with asymptomatic rotavirus infection, and are therefore likely to have under-estimated SAR for infection.

The only data on household rotavirus transmission from middle income countries comes from two studies from Ecuador and India. Both were conducted in household contacts of childhood rotavirus cases, and described strikingly different SAR between populations. Ecuador reported a SAR for infection of 55%(106), while India reported a SAR for infection of 0.54%(268). Whilst both of these studies used qRT-PCR to detect rotavirus in stool samples from household contacts, in India samples were screened first using EIA to detect rotavirus antigen. EIA will only detect large quantities of virus in the stool, broadly corresponding to those amounts typically found in association with clinical disease rather than with asymptomatic infection, so this may partly explain the observed discrepancy in attack rates.

There are no data describing household transmission of rotavirus from LIC or from sub-Saharan Africa. Findings from low and middle income settings cannot be generalised to low income, sub-Saharan countries as factors which may have a major influence on the risk of transmission such as living environments, crowding, contact patterns, access to sanitation systems and frequency of exposure to rotavirus are fundamentally different from those in high income and middle income settings. We therefore aimed to describe SAR for rotavirus infection and disease in household members after contact with a symptomatic rotavirus case in an urban setting in Malawi.

5.2 Methods

5.2.1 Overview

This chapter, and the following 3 chapters, describe data collected as part of the RotaRITE transmission epidemiology study (RRTE), conducted in Blantyre, Malawi.

5.2.2 Objectives

1. To define SAR for rotavirus infection to household contacts of a symptomatic index child.
2. To define SAR for rotavirus disease to household contacts of a symptomatic index child.
3. To describe rotavirus genotypes in symptomatic index children and relate this to rotavirus genotypes identified in household contacts

5.2.3 Study design

This was a prospective cohort study initially designed to investigate the potential impact of rotavirus vaccination of infants on SAR within households. The study identified index children with acute rotavirus gastroenteritis and followed up members of their households for asymptomatic rotavirus infection and clinical rotavirus disease.

5.2.4 Study site

This study was conducted at Queen Elizabeth Central Hospital (QECH), and three government health centres in Blantyre: Zingwangwa, Gateway and Madziabango.

5.2.5 Study population

The RRTE study recruited households of vaccine-age eligible children presenting with rotavirus positive gastroenteritis (index cases) to four government health facilities in Blantyre; QECH, Zingwangwa Health Centre, Gateway Health Centre, and Madziabango Health Centre. Eligibility criteria for Index cases and household members are outlined below. Recruitment at QECH commenced on the 16th February 2015, and at Zingwangwa one month later to allow for training of study staff. Gateway Health Centre, an adjacent facility to QECH, was subsequently added as an additional site to ensure adequate representation of the mild and moderate cases of rotavirus AGE presenting to QECH. This was because on starting the study it became apparent that some children with milder diarrhoea were referred from QECH to Gateway Clinic. Madziabango was added as fourth site in August 2016 as an attempt to increase recruitment of unvaccinated children, as there had been a recent vaccine stock out in the area. Locations of these facilities can be seen in the map in Figs 2.3 and 2.4 in Chapter 2 (pages 84 and 85). The monovalent rotavirus vaccine (RV1) was incorporated in to the Malawi Expanded Programme on Immunisation (EPI) EPI schedule in Malawi on 29th October 2012. Two oral doses are given at 6 and 10 weeks.

5.2.6 Integration with other studies

As described in Chapter 2, section 2.1, page 76, the RotaRITE study was made up of two complementary but distinct study arms. This study makes up one arm of the RotaRITE study (RotaRITE Transmission Epidemiology, the RRTE study), the second arm (RotaRITE: response to immunization, the RRRI study) investigated mechanisms underpinning rotavirus vaccine failure and was undertaken by Dr. Louisa Pollock.

At QECH, RotaRITE was nested within an existing diarrhoeal surveillance platform, established to monitor rotavirus vaccine effectiveness ("New Childhood Vaccines for Malawi" (VacSurv) NHSRC #867) [PI Professor Nigel Cunliffe]. Children recruited by the surveillance platform were assessed for eligibility to participate in the RotaRITE study. Dependent on vaccination status and results of rotavirus diagnostic tests, children were eligible for one or both of the RotaRITE study arms. Consent and data collection processes are described in detail below in section 5.2.7. In the health centres no diarrhoeal

surveillance platform was in place. The RotaRITE study therefore identified and recruited eligible children directly.

5.2.7 Study procedures

5.2.7.1 Identification of participants for diarrhoeal surveillance platform at QECH

Recruitment took place during the routine working hours of 8am and 4pm Monday to Friday from February 16th 2015 to 11th November 2016. Clinicians and nurses working in A&E at QECH were asked to refer all children with diarrhoea to the study team and study research nurses based in A&E actively screened waiting patients for diarrhoeal cases. Admission records and the paediatric wards were also screened on a daily basis to identify all cases admitted with gastroenteritis. At the end of each day admission books were checked for the number of children with diarrhoea and compared to the number screened. The number of children not screened was documented in screening logs. This study was originally powered to investigate the difference in proportion of household members shedding wild-type rotavirus in households where the index child was vaccinated, in comparison to households where the index child was not vaccinated and to run over a 24 month period however due to the success of the rotavirus vaccine programme in Malawi it was not possible to identify unvaccinated children and recruitment was stopped early in November 2016 (Section 5.3.8).

Cases were eligible for enrolment in the surveillance study if they met the ALL following criteria:

- <5 years of age.
- Lived in Blantyre district
- Presented to QECH with diarrhoea
- Clinical illness not explained by an alternative underlying medical condition
- Clinical illness commenced within 14 days prior to hospital visit; and
- Either:
    - Seen at A&E at QECH and treated with rehydration (oral or intravenous) for diarrhoea and discharged home; or
    - Admitted to the hospital and treated for diarrhoea

Cases were not eligible for enrolment in the surveillance study if they met ANY of the following criteria:

- Unable to contact parent or guardian to obtain informed consent

- Admitted to another hospital for >24 hours (and subsequently transferred to QECH)
- Re-presentation within 14 days of previous hospital separation for the same illness
- Hospital admission >48 hours prior to enrolment
- Known oncological diagnosis or congenital immunodeficiency (apart from HIV infection)

Families of eligible children were invited to participate, and if in agreement, written informed consent obtained. A combined enrolment form was used for all 3 studies, and a single bulk stool sample collected.

5.2.7.2 Identification of potential participants for the RotaRITE studies at QECH

Vaccine age eligible children (born on or after 17th September 2012) enrolled in the surveillance platform had a rotavirus rapid immunochromatographic test (ICT) performed on stool samples, and depending on the result of this underwent further eligibility screening for the RotaRITE studies.

5.2.7.3 Initial screening at health centres

Clinical officers and nurses at participating health centres were asked to refer any child who presented with diarrhoea to the study team. The study team also surveyed the clinic for children with diarrhoea. At the end of each day the clinic admissions books were checked for the number of children with diarrhoea and compared to the number screened. The children not screened were documented in the screening log, together with the reason for not screening.

Parents/guardians of vaccine age eligible children presenting with diarrhoea were approached by the study team and invited to take part in a screening step for the RotaRITE studies. Following informed consent a stool sample was obtained, and an ICT test performed. Rotavirus positive children fulfilling eligibility criteria were then enrolled into the RRTE study as index cases following informed consent from their parent or guardian.

5.2.7.4 Eligibility Criteria for Index cases for RR TE Study

Infants were eligible for enrolment as rotavirus positive index children if they met ALL the following criteria:

- Presented to health care facility with diarrhoea after onset of study
- Clinical illness commenced within 7 days of hospital visit
- Age eligible to have received rotavirus vaccine (i.e. born on or after 17th September 2012)

- Aged 6 weeks old or older
- Produced a stool sample positive for rotavirus on IC rapid test during the first 72 hours after presentation

Infants were excluded from enrolment as a rotavirus positive index children if ANY of the following applied:

- Live outside Blantyre district
- Re-presentation within 14 days of previous admission for the same illness
- Admission >72 hours prior to enrolment
- Had received only a single dose of rotavirus vaccine (RV1)
- Received RV1 within the last 14 days
- Lived in an institution

5.2.7.5 Study procedures at enrolment of index child

Study procedures at enrolment are summarized in box 1.

History taking

Data was collected from the parent/guardian of the index child on presenting symptoms and their duration, past medical history including HIV status, vaccine history and the number of people in the household. Data were collected using a questionnaire and from medical records and health passports. Health passports are hand held government issued health records which document vaccination, and attendances to health care facilities.

Box 1.

Overview of assessment of index child at point of recruitment:

- Vaccination status – confirmed by health passport
- Age eligibility
- HIV testing
- Serum rotavirus IgA levels
- Nutritional assessment (mid upper arm circumference (MUAC), weight and height)
- Vesikari scoring for disease severity [1]
- Stool viral load (assessed using real-time qRT-PCR
- Questionnaire covering symptoms, past medical history and demographic data

Clinical assessment

Children had their weight, height and mid-upper arm circumference measured according to a standardized protocol. Disease severity was assessed using the Vesikari score, a 20 point clinical scoring system for gastroenteritis. This is outlined in Table 5.1. The clinical

definition of some and severe dehydration used in the Vesikari score is also outlined in Table 5.2.

Table 5.1. Components of the Vesikari score.

Category	Values		
Maximum number of loose stools per day	1-3	4-5	≥6
Diarrhoea duration (days)	1-4	5	≥6
Maximum number of vomits per day	1	2-4	≥5
Vomiting duration (days)	1	2	≥3
Temperature	35.9-37.3	37.4-37.8	≥37.9
Dehydration	No dehydration	Some dehydration	≥6% severe dehydration
Treatment	Oral rehydration	Hospitalization for >24h or IV rehydration	n/a
Score awarded	1	2	3
TOTAL SCORE			
Severity Category	<7 mild	7-10 moderate	≥11 severe

Table 5.2. Defining dehydration for use in Vesikari score

Signs of Dehydration	Dehydration Classification
Treated with Plan C or Two signs of: • Sunken eyes • Lethargic/unconscious • Not able to drink/drinking poorly • Skin pinch goes back very slowly	Severe dehydration (≥ 6% dehydration) Vesikari score 3 for dehydration
Two signs of: • Sunken eyes • Restless/irritable • Thirsty/drinks eagerly • Skin pinch goes back slowly OR one severe dehydration and one some sign	Some dehydration (1-5% dehydration) Vesikari score 2 for dehydration
Does not meet criteria for Plan C or Plan B	No dehydration Vesikari score 1 for dehydration

Where plan B and C are WHO plans for rehydration, plan C involving rapid intravenous rehydration and plan B oral rehydration.

5.2.7.6 Sample collection at recruitment of the index child.

Stool samples

Bulk stool samples were collected preferentially. Rectal swabs were also used to collect stool samples from index children where it was not possible to obtain a bulk stool sample. Rectal swabs were introduced in September 2015 in response to a concern that children with milder disease were being under-ascertained because of inability to obtain a bulk stool sample.

Blood samples

1-2 mls of venous blood was collected from index children at presentation.

HIV testing

All children attending health care facilities were offered HIV testing in line with current Malawi National Guidelines using ELISA point of care tests with confirmatory HIV-PCR if aged under 12 months of age. HIV testing services were those provided by the Ministry of Health. Study staff encouraged and facilitated the testing of children and their guardians, but did not themselves actively conduct HIV testing.

5.2.7.7 Household visits and follow up

After initial recruitment of the index child a preliminary home visit took place. The household head or their representative was asked for consent for the household to participate. All adult household members were then individually consented and assent was obtained from age appropriate children. Procedures at household visits are outlined in Table 5.3.

5.2.7.8 Eligibility criteria for household contacts for RRTE Study

Individuals were eligible for recruitment as household contacts of rotavirus positive index children if they met ALL the following criteria:

- Live in household containing a rotavirus positive index child
- Identify with same household head as index child
- Have lived in household for at least 3 weeks prior to enrolment
- Able to perform household visit within 10 days of onset of symptoms in index child

Individuals were excluded from enrolment as a household contact of a rotavirus positive index patient if ANY of the following applied

- Live outside Blantyre district

- Unable to contact household head or their representative to obtain informed consent

5.2.7.9 Procedures at initial household visit

History taking

Field workers collected data on past medical history including HIV status, current symptoms and contact patterns with the index child for each household member. Vaccine history was collected from children under the age of 5 years.

Clinical assessment

Any children under 5 years had their weight, height and mid-upper arm circumference measured and documented.

Documentation of household location

Household location was documented using Global Positioning System (GPS) software.

Sample collection

At the end of the initial visit field workers left sample containers for each study participant in the household. These were clearly labelled to identify which container should be used for each participant.

5.2.9.10 Follow up household visits

The initial visit was followed by two additional visits to collect stool samples. The first stool sample aimed to be 5-7 days after the onset of symptoms in the index child and the second stool sample 10-12 days after the onset of symptoms in the index child (Fig 5.1). Brief data were collected on symptoms in each household member since the last visit.

Table 5.3. Overview of processes to be carried out at each household visit

	Initial visit	Visit 1	Visit 2
Recruitment of household members	✓		
Nutritional assessment of household members aged under 5 years	✓		
HIV status of mothers and other children (from health-care records)	✓		
Confirmation of vaccine status (children)	✓		
Demographic Questionnaire	✓		
Symptom Questionnaire		✓	✓
Containers left for stool sample	✓	✓	
Collection of stool sample		✓	✓

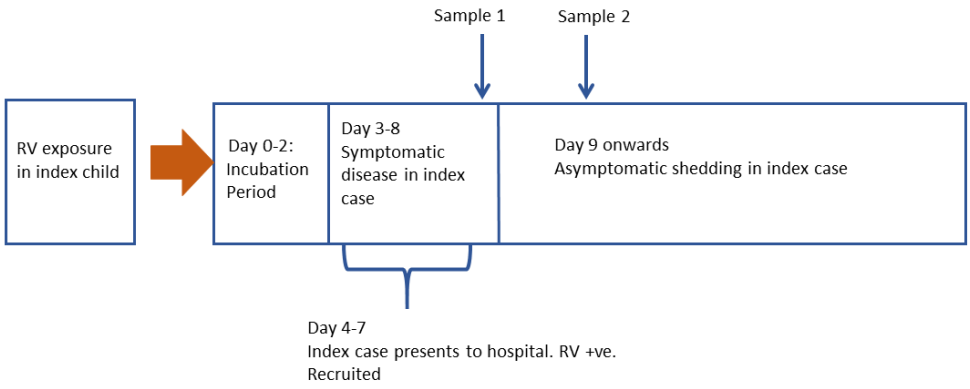


Figure 5.1 Overview of timing of stool samples

5.2.7.11 Control recruitment

On interim analysis of stool samples from household contacts of rotavirus positive index cases 50% of household contacts were found to have detectable rotavirus in at least one stool sample. In light of this high frequency of detectable rotavirus a decision was made to recruit 55 control households to describe the frequency of rotavirus shedding in household members without history of recent exposure to a rotavirus case. Control households were selected to contain a child retrospectively frequency matched to the age distribution of rotavirus positive index children already recruited into RRTE. Random GPS locations within the Blantyre Municipality were generated using R software (Version 3.3.2). Households at each random location were visited, moving outwards in a systematic

manner until suitable control patients were found and recruited. Household members of control households were asked to complete a CRF covering symptom and demographic data, and to produce a single stool sample. Control households were recruited between July 2016 and January 2017.

5.2.7.12 Eligibility criteria for control households

Households were eligible for enrolment as control households if ALL of the following applied

a) Household contained a child whose age was frequency matched to a RRTE index case child

b) No one resident in the household had gastroenteritis symptoms at the time of recruitment or in the 2 weeks preceding

Households were excluded from enrolment as control households if

a) Household contained a known rotavirus case

5.2.7.13 Consent procedures

Informed consent was obtained for each stage of the recruitment process, with parental consent obtained for children. In addition to parental consent, assent was sought from children aged 8 years and above. Initially written assent was obtained from all children, but these procedures were revised part way through the study following feedback from the field team in order to minimize the burden to the family. From December 2015 onwards children aged 8-11 underwent a verbal assent process, while children aged 12 years and over continued to provide written assent. For illiterate participants a thumb print witnessed by an independent party was used to confirm consent.

5.2.8 Sample size calculations

5.2.8.1 Primary study

This study was powered to compare the proportion of household members shedding rotavirus in households were the index child was vaccinated, compared to households were the index child was unvaccinated. The sample size was inflated to account for clustering at the household level based on an estimate of the intra-class correlation co-efficient (ICC) of 0.5, derived from a study of household rotavirus transmission in Ecuador. Under the following assumptions an initial sample size of 306 was selected

- Precision of 0.05%

- Power of 80%
- ICC 0.5
- Household size of 5
- Baseline transmission rate 50% in unvaccinated households,
- Transmission rate of 37.5% (25% reduction) in vaccinated households

An interim analysis by an independent statistician after 6 months of recruitment revised the estimate of the ICC based on interim RRTE data to 0.13 (95% CI 0.03, 0.42) and prevalence of shedding in vaccinated household contacts to 55%. The target sample size was subsequently revised to 182 households; 146 households containing a vaccinated child, and 36 containing an unvaccinated child.

5.2.8.2 Control households

55 control households was selected to allow detection of a significant difference in the proportion of household members shedding rotavirus in control households, compared to households exposed to an index case given the following assumptions:

- Precision of 0.05
- Power of 80%
- ICC of 0.3
- Household size of 5
- Rotavirus shedding in 31 % of unexposed household members (ie asymptomatic control households)
- Rotavirus shedding in 49% of exposed household members (ie household contacts of a rotavirus case.

The proportion of asymptomatic household members expected to be shedding was based on the proportion of asymptomatic control children found to be shedding rotavirus on qRT-PCR from a case-control study in Malawi(20). The proportion of exposed household members shedding was based on interim analysis of RRTE data.

5.2.9 Laboratory procedures

Laboratory procedures are described in detail in chapter 2, section 2.3, page 87. With the exception of ICT rapid tests for rotavirus in stool, all laboratory procedures were conducted in the research laboratories at the Malawi-Liverpool-Wellcome Trust clinical research programme.

5.2.9.1 Laboratory tests at recruitment for index children

Stool samples

ICT rapid tests. Stool samples were tested for rotavirus antigen in real time using ICT rapid tests to determine eligibility for the RRTE study. This was undertaken at the clinical site of recruitment by the study nurses.

Enzyme-immunoassay (EIA) tests. Children recruited into the Vacsurv surveillance platform had stool samples tested with EIA in addition to qRT-PCR and IC tests. This was for with historical surveillance, and in line with WHO guidance for rotavirus surveillance. EIA results were not used in the RRTE study except as a quality control to compare the sensitivity and specificity of IC tests for rotavirus.

Molecular tests: Stool samples from all index children recruited into the RRTE study were tested using real-time qRT-PCR (RT qRT-PCR) to assess stool viral load and by qualitative RT-PCR to determine genotype.

Blood samples

Anti-rotavirus IgA titres: Serum was stored for testing for anti-rotavirus IgA titres to assess pre-existing immunity to rotavirus at a later date.

5.2.9.2 Laboratory procedures for household follow up visits

Stool samples

Molecular tests: Stools collected from household visits were tested for rotavirus using real-time qRT-PCR. Rotavirus positive samples with a Ct value of <=35 underwent qualitative RT-PCR to determine genotype. Only one sample from any one individual underwent genotyping. Any samples positive for rotavirus but where the Ct value was >35 underwent a confirmatory PCR for a second target (NSP3).

5.2.10 Statistical methods

Distributions of continuous variables were examined and categorical variables were tabulated to generate descriptive statistics. Missing observations were excluded from analysis. Two-sided t-tests were used to compare independent means of normally

distributed data and rank sum tests were used to compare non-normally distributed data. Chi squared or Fischers exact tests were used to compare categorical variables, depending on the number of observations present.

5.2.11 Managing specific variables

The question of whether or not the household contact was responsible for changing the index child's nappy was only asked to adults in the household. To enable inclusion of this variable in multi-variable models without problems of sparse data, a dummy variable was created for "child" where the response for nappy changing was "unknown". Similarly children under 16 were not asked if they were the primary care giver for the child. For this variable, it was assumed that children under 16 were not primary care givers, given that there was an additional adult in the household and in all but one of our recruited households a mother was present.

Wealth

Individual proxy variables for poverty were compared between groups. In addition, a composite variable was generated to rank participants against each other in terms of relative wealth using a modified version of model developed by Payongyong et a using the 1998 Malawi DHS data(340). The coefficients for this model are given in Table 5.4

Table 5.4. Coefficients for proxy means test model developed by Payongyong et al from the Malawi 1998 DHS.

Preferred urban Malawi proxy means test model	
Dependent variable: log household welfare indicator	
Response variable	Co-efficient
HH owns a fridge	0.518
Household size	-0.306
Household size squared	0.016
Age of head of household	0.005
Education level of household head	0.151
No. of salaried HH members	0.061
HH owns a motor vehicle	0.704
HH get lighting from electricity or gas	0.280
HH owns a bed	0.247
Blantyre City	-0.037
Constant	2.347
Observations	*872*
R-squared	*0.60*

Reproduced from *Payongayong E, Benson T, Ahmed A, Kanyanda C, Mwanza P, Chilopa K, Banda N MA. Simple household poverty assesment models for Malawi: Proxy Means Test from the 1997–98 Malawi Integrated Household Survey. 2006.* The model was modified by substituting car or vehicle for mobile phone which was felt to be more relevant to current poverty levels. A continuous variable for wealth was generated by multiplying the identified variables by their respective co-efficient. This was then split into quintiles.

Nutritional status

Admission weights in index children were adjusted to account for dehydration by multiplying by 110% for children with severe dehydration and 105% for children with some dehydration. These weights were used to calculate Z Scores, referred to as adjusted Z scores in the text. Nutritional status was defined using WHO standards, as described in Chapter 4, section 4.2.5, page 130. Weights were not adjusted for community controls or household contacts of index children.

HIV status

HIV exposure and infection was defined as described in Chapter 4, section 4.2.5, page 130.

5.2.12 Ethics

The RRTE study was reviewed and approved by the University of Liverpool Research Ethics committee (# 000757), and the Malawi College of Medicine Research Ethics Committee (P.09/14/1623). Sponsorship was provided by the University of Liverpool. Ethical approval for the diarrhoeal surveillance platform was provided by the University of Liverpool Research Ethics Committee (# 000490) and by the National Health Sciences Research Committee, Lilongwe, Malawi (# 867)

5.2.13 Defining of outcome variables

Defining rotavirus positivity for infection secondary attack rates

Results of rotavirus VP6 qRT-PCR are presented as copy numbers or log transformed copy numbers. Samples with a Ct value in range 35-40 underwent NSP3 PCR as a confirmatory assay. Due to lack of reproducibility in samples with very low viral loads samples were defined as rotavirus positive if they had >=100 viral copy numbers and were positive on NSP3 assay.

Defining clinical disease for disease secondary attack rates

Clinical disease in household contacts was defined as any reported vomiting or diarrhoea in individuals who tested rotavirus positive on at least one stool sample. This is a broader definition than used to define diarrhoea in index children where >=3 loose stools in 24 hours was used. This was to increase sensitivity of detection of clinical disease in household contacts.

5.2.14 Other definitions

Vaccine age eligible: born on or after 17th September 2012.

Index child: vaccine age-eligible household member with rotavirus positive gastro-enteritis, presenting to a health facility

Shedding density: viral copy number, derived from Ct value of real-time PCR.

Severe rotavirus disease: Rotavirus positive AGE with Vesikari scale $\geqslant$ 11 [41]

Mild to moderate disease: Rotavirus positive AGE with Vesikari scale of 1-10.

Asymptomatic infection: Rotavirus detected by PCR but no diarrhoea or vomiting reported during follow-up, or in the preceding 10 days.

Household: group of individuals who identify with the same household head, and live within the same physical structure as each other.

Household contact: an individual living in the same household as the index child and fulfilling eligibility criteria for the study

5.3 Results

5.3.1 Description of cohort

Recruitment for the primary study took place from February 16th 2015 to 11th November 2016. A total of 196 households were recruited into the study.

Breakdown of screening and recruitment by site can be seen in Fig. 5.2.

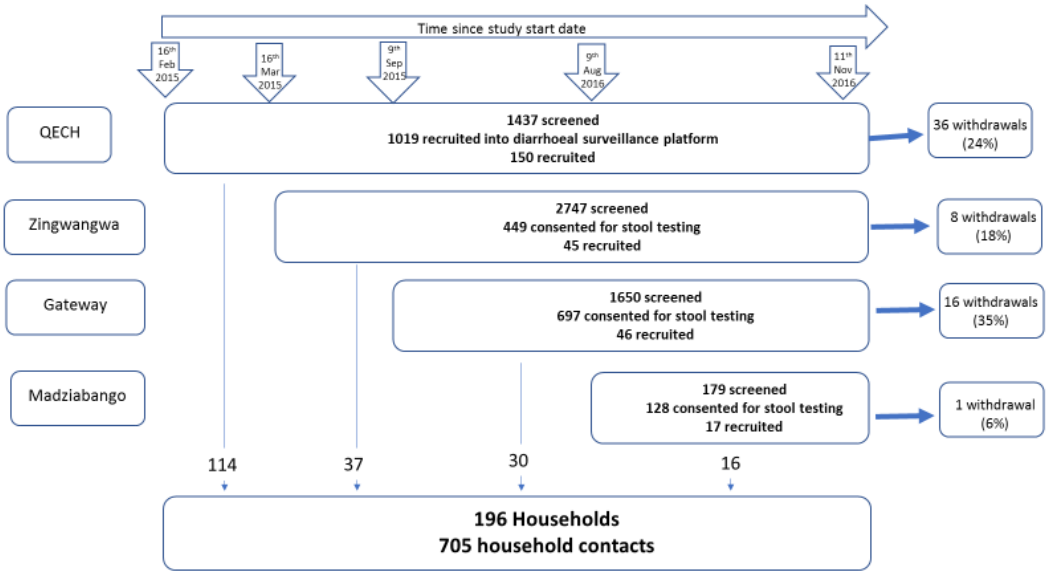


Figure 5.2 Overview of RRTE study recruitment

5.3.2 Description of index children

Characteristics of the rotavirus positive index children recruited are listed in Table 5.5. Median age was 11.5 months (IQR 8.8, 15.2). There was a slight preponderance to male sex in rotavirus positive index children. Approximately 13% of children were HIV exposed (25/196), and of those with data available 2/58 (3.5%) were HIV infected.

The majority of children had severe rotavirus gastroenteritis, as defined as a Vesikari score ≥11 (168/193, 86.5%). Vomiting was a prominent symptom, with 182/196 (92.9%) reporting at least one episode. Most had at least some clinical evidence of dehydration (124/196, 63.3%), and around one quarter were severely dehydrated (46/196, 23.5%). Approximately one third required intravenous (IV) rehydration (58/196, 29.6%), and over half were admitted to hospital (111/196, 56.6%). A total of 2/196 (1.02%) children recruited as index cases died. Children were typically underweight with a mean adjusted weight-for-height (WHZ) Z score of -0.59 (standard deviation [SD] 1.61). Mean height-for age Z score (HAZ) was -0.04 (SD 2.46). Prevalence of severe acute malnutrition after

adjusting for dehydration was 23/194 (11.9%). Previous history of diarrhoeal disease was common, with almost half of the index children having attended a health care facility for diarrhoeal disease on a previous occasion (91/196, 46.4%). Breast feeding was almost universal (195/196, 99.5%). Rotavirus vaccine coverage was very high at 194/196 (99.0%).

113 of 196 (57.7%) children were recruited at QECH, and the remainder were recruited from health centres. Children recruited at QECH had more severe disease than those recruited at health centres (median Vesikari score 15 (IQR 14, 16) vs 12 (IQR 10,14) p<0.001) (Table A3, appendix, page 261). Children recruited at QECH also tended to be more wasted (mean WHZ -1, SD 1.5) compared to children from health centres (mean WHZ -0.34, SD 1.7, p=0.005). Significantly more children recruited from QECH had had a previous presentation to a health facility with diarrhoeal disease (69/113 [61.1%] vs 22/83 [26.5] p<0.001). There were no significant differences in terms of feeding, birth weight, age, sex or vaccine status between groups.

Table 5.5. Description of index children in primary study, and control children

	Primary study Summary statistic	Missing data	Controls Summary statistic	Missing data	P value†
Age (median and IQR)	11.5(8.8,15.2)	0/196	11.5(8.2,15.4)	0/55	0.75**
Sex (male) (%)	108/196 (55.1)	0/196	26/55(47.3)	0/55	0.304
Diarrhoea (%)	196/196 (100)	0/196	0/55 (0)	0/55	<0.001
Duration (days)		0/196			
1-3 (%)	173/196 (88.3)		-	-	-
5	12/196 (6.1)		-	-	-
≥6	11/196 (5.6)		-	-	-
Episodes(n)***		0/196			
1-4 (%)	24/196 (12.2)		-	-	-
5	86/196 (43.9)		-	-	-
≥6	86/196 (43.9)		-	-	-
Vomiting (%)	182/196 (92.9)	0/196	0/55 (0)	0/55	<0.001
Duration (days)		0/182			
1 (%)	23 (12.6)		-	-	-
2	59 (32.4)		-	-	-
≥3	100 (55.0)		-	-	-
Frequency (n)		0/182			
<5 (%)	123 (67.6)		-	-	-
≥5	59 (32.4)		-	-	-
HIV					
Exposed (%)	25/196 (12.8)	0/196	6/54 (11.1)	1/55	0.75
Infected (%)	2/58 (3.5)	138/196*	0/11 (0)	44/55	0.53
Completed rotavirus vaccination (%)					
Vaccinated (2 doses)	194/196 (99.0)	0/196	55/55 (100)	0/55	0.45

Unvaccinated (0 dose)	2/196 (1.0)	0/196	0/55 (0)	0/55	0.45
Admitted (%)					
Yes	111/196 (56.6)	0/196	-	-	-
Vesikari score (IQR)	14 (12, 16)	3/196	-	-	-
Temperature (rectal, ^{o}C)					
37.1-38.4 (%)	92/193 (47.7)		-	-	-
38.5-38.9	48/193 (24.9)		-	-	-
≥39.0	53/193 (27.5)		-	-	-
Thirst (%)					
No thirst	32/196 (16.3)		-	-	-
Thirsty	141/196 (71.9)		-	-	-
Drinks poorly	23/196 (11.7)		-	-	-
Skin pinch (%)					
Normal	56/196 (28.6)		-	-	-
Goes back slowly	104/196 (53.1)		-	-	-
Goes back very slowly	36/196 (18.4)		-	-	-
General Appearance (%)					
Well, alert	94/196 (48.0)		-	-	-
Restless	83/196 (42.4)		-	-	-
Unconscious	19/196 (9.7)		-	-	-
Dehydration (%)					
None	26/196 (13.3)		-	-	-
Some (5%)	124/196 (63.3)		-	-	-
Severe (10%)	46/196 (23.5)	0/196	-	-	-
IV fluids (%)					
Yes	58/196 (29.6)	0/196	-	-	-
Oral fluids (%)					
Yes	185/196 (94.4)	0/196	-	-	-
Outcome (%)					
Home	194/196 (99.0)		-	-	-
Died	2/196 (1.0)	0/196	-	-	-
Anthropometry, mean (SD)					
Adjusted WHZ	-0.59(1.61)	1/196	-	-	-
Adjusted WAZ	-0.46 (1.6)	1/196	-	-	-
Adjusted HAZ	-0.04 (2.46)	5/196	-	-	-
MUAC	13.48 (1.28)	1/196	-	-	-
SAM	23/194 (11.9)	3/196	-	-	-
Previous diarrhoeal admission (%)	15/196 (7.7)	0/196	3/55 (5.5)	0/55	0.58
Previous diarrhoeal presentation (%)	91/196 (46.4)	0/196	26/55(47.3)	0/55	0.912
Premature (%)	7/196 (3.6)	0/196	2/53 (3.6)	0/55	0.83
Birth weight, mean (SD)	2.96 (0.63)	12/196	2.95(0.55)	4/55	0.99*
Ever Breastfed (%)	195/196 (99.5)	0/196	2/55 (3.6)	0/55	0.06
Diet includes food other than breast milk (%)	190/196 (97.0)	0/196	48/55(87.3)	0/55	0.004

†P values are X^2 p values for differences in proportions between case children and control children unless otherwise specified. *2 sided independent ttest **rank sum test. Clinical data not collected for control children

5.3.3 Description of households

Characteristics of the households of index children are listed in Table 5.6. Median household size was 5 (IQR 3, 6). Just under 50% of households had electricity at home (89/196 (45.4%), and the majority shared a toilet with at least one other household (148/196, 75.5%). Over one third of households took over 30 mins to access water (68/196 [34.7%]) and most households sourced water from a shared tap to their village or compound (115/195 [59.0%]). In most households at least one person had a regular salary (127/196 [65.1%]). Nearly one third of households families reported sometimes having difficulty getting the food they need (60/196 [30.6]), and almost a quarter 45/196 [23.0%] reported an adult missing a meal in the last two weeks to ensure that the other family members had enough to eat. Recruitment of households was reasonably equally distributed throughout the year. 114/196 (57.9%) were recruited in the rotavirus season, where the season was defined as May to October.

Table 5.6. Description of households in primary study, and control households

	Primary study Summary statistic	Missing data	Controls Summary statistic	Missing data	P value
Household size (%)		0/196		0/55	
≤5	136/196(69.4)		44/55 (80.0)		
>5	60/196 (30.6)		11/55 (20.0)		0.123
Additional child <1 (%)					
0	187/192(97.4)		54/55 (98.2)		
1	5/192 (2.6)	4/196	1/55 (1.8)	0/55	0.739
Additional children < 5 (%)					
0	126 (65.0)		43/55 (78.2)		
1	62 (32.0)		10/55 (18.2)		
2	5 (2.6)		2/55(3.6)		
4	1 (0.5)	2/196	0/55 (0)	0/55	0.224
Education level of mother (%)					
Primary or less	108/196(55.1)		24/55 (43.6)		
Secondary	81/196 (41.3)		29/55 (52.7)		
Higher	7/196 (3.6)	0/196	2/55 (3.6)	0/55	0.309
Education level of household head (%)					
Primary or less	59/192 (30.7)		14/55 (25.5)		
Secondary	113/192(58.9)		35/55 (63.6)		
Higher	20/192 (10.4)	4/196	6/55 (10.9)	0/55	0.750
Electricity at home					
Yes (%)	89/196 (45.4)	0/196	27/55 (49.1)	0/55	0.628
Shared toilet					
Yes(%)	148/196(75.5)	0/196	40/55 (72.7)	0/55	0.674
How long for household to access water (%)					
0-5 mins	34/196 (17.4)		22/55 (40.0)		
5-30mins	94/196 (48.0)		30/55 (54.6)		
>30 mins	68/196 (34.7)	0/196	3/55 (5.5)	0/55	<0.001

Water source (%)					
Well	16/195 (8.2)		3/55 (5.5)		
Borehole	35/195 (18.0)		11/55 (20.0)		
Shared tap	115/195(59.0)		38/55 (69.1)		
Tap to house	29/195 (15.0)	1/196	3/55 (5.5)	0/55	0.003
How many people have a regular salary (%)					
0	68/195 (34.9)		5/55 (9.1)		
≥1	127/195(65.1)	1/196	50/55 (90.9)	0/55	0.001
Problems getting food in the past month (%)					
No	136/196(69.4)		26/55 (47.3)		
Sometimes/often	60/196 (30.6)	0/196	29/55 (52.7)	0/55	0.002
Has an adult skipped a meal in the past 2 weeks?					
Yes (%)	45/196 (23.0)	0/196	10/55 (18.2)	0/55	0.449
Wealth indicator (mean and SD)	2.38 (0.57)	5/196	2.55(0.52)	1/55	0.040
Time of recruitment					
Quarter of year (%)					
Jan-Mar	43/196 (22.0)		3/55 (5.5)		
Apr-Jun	58/196 (29.6)		0/55 (0)		
Jul-Sept	56/196 (28.6)		28/55 (50.9)		
Oct-Dec	39/196 (19.9)	0/196	24/55 (43.6)	0/55	<0.001
Season (%)					
In season	114/196(57.9)		27/55 (49.1)		
Out of season	82/196 (42.0)	0/196	28/55 (50.9)	0/55	0.337

[†]P values are X^2 p values for differences in proportions between case children and control children unless otherwise specified. *2 sided independent ttest **rank sum test

5.3.4 Description of household members

705 household members of 196 index children were recruited. Characteristics of these household members can be found in Table 5.7. The median age of household recruits was 19 years, ranging from 4 months to 61 years. Household recruits were slightly more likely to be female (386/702 [55.0%]) than male. Half of the household recruits had been tested for HIV (345/665 [51.8%)], and of those with available results 29/338 were HIV infected (8.6%). The majority of household recruits were children, (312/705 [44.3%], followed by mothers (195/705 [27.7%]) and then other adults (198/705 [28.1%]).

For household contacts aged under 5 years, 8/89 (9.0%) reported a previous hospital attendance with diarrhoeal disease. Mean WHZ was lower than the WHO standard at -0.24 (SD 1.9), and children were stunted, with a mean HAZ of -1.55 (SD 1.5), Prevalence of SAM was 6/75 (8.0).

Most household members slept in the same room as the index child (423/705 [60.0%]), with half sharing a bed (359/705 [50.9%]). Just over 40% of contacts spent all day in the house (296/705 [41.9%]), and the same number spent all day with the index child. Most

people used a simple pit toilet or ventilated improved pit latrine (VIP), (659/705[93.5%]). Most people did not share a toilet with the index child (684/705 [97.0%]) – which is likely to reflect the fact that most index children were infants who would not yet use a toilet. 209/705 [29.7%] were identified as the primary carer for the index child, and a third were identified as responsible for changing the index child's nappy (203/705 [28.8%]).

Table 5.7. Description of house contacts in primary study, and control household contacts

Variable	Primary study Summary statistic	Missing data	Controls Summary statistic	Missing data	P value
Household member age (%)					
<5 years	92/702 (13.1)		11/153 (7.2)		
5-15 years	198/702 (28.2)		53/153 (34.6)		
15-45 years	394/702 (56.1)		87/153 (56.9)		
45+ years	18/702 (2.6)	3/705	2/153 (1.3)	0/153	0.099
Sex (male) (%)	316/702 (45.0)	3/705	54/153 (35.3)	0/153	0.028
Diarrhoea (%)	40/705 (5.7)	0/705	0/153 (0)	0/153	0.003
Vomiting (%)	17/705 (2.4)	0/705	0/153 (0)	0/153	0.052
HIV					
Ever tested (%)	345/665 (51.8)	40/705	86/149 (57.7)	4/153	0.197
HIV Infected (%)	29/338 (8.6)	7/345	6/86 (7.0)	0/86	0.630
Relationship to child (%)					
Mother	195/705 (27.7)		55/153 (36.0)		
Other adult relative	198/705 (28.1)		32/153 (20.9)		
Child contact	312/705 (44.3)	0/705	66/153 (43.1)	0/153	0.067
Contact behaviour					
Sleep in same room as child (%)	423/705 (60.0)	0/705	88/153 (57.5)	0/153	0.570
Share a bed with index child (%)	359/705 (50.9)	0/705	82/153 (53.6)	0/153	0.549
Time spent in house (%)					
All day	296/705 (42.0)		74/153 (48.4)		
Half day	244/705 (34.6)		52/153 (34.0)		
Evening only/no time	165/705 (23.4)	0/705	27/153 (17.7)	0/153	0.216
Time spent with index child (%)					
All day	296/705 (42.0)		74/153 (48.4)		
Half day	243/705 (34.5)		52/153 (34.0)		
Evening only/no time	166/705 (24.0)	0/705	27/153 (17.7)	0/153	0.208
Share toilet with index child (%)					
Never	684/705 (97.0)		130/153 (98.3)	0/153	
Sometimes	16/705 (2.3)		2/153 (1.3)	0/153	
Often	3/705 (0.4)		0/153 (0.0)	0/153	
Always	2/705 (0.3)	0/705	1/153 (0.7)	0/153	0.634
Toilet type (%)					
None	13/705 (1.8)		3/153 (2.0)		
Simple pit/VIP	659/705 (93.5)		143/153 (93.5)		
Water toilet	33/705 (5.0)	0/705	7/153 (4.6)	0/153	0.994
Primary care giver for index child					
Yes (%)	209/705 (29.7)	0/705	56/153 (36.6)	0/153	0.091
Responsible for changing nappy					
Never/sometimes (%)	190/705 (27.0)	0/705	34/153 (22.2)	0/153	
Always/often	203/705 (28.8)	0/705	53/153 (34.6)	0/153	
N/A	312/705 (44.3)	0/705	66/153 (43.1)	0/153	0.279
Under 5s only					
RV1 doses (%)					

0 doses	36/79 (45.6)		2/10 (20.0)		
1 doses	2/79 (2.5)		0/10 (0.0)		
2 doses	41/79 (51.9)	12/91	8/10 (80.0)	1/11	0.236
Previous history of clinic visit with diarrhoea (%)					
No	80/89 (89.9)		11/11 (100)		
Yes	8/89 (9.0)		0/11 (0)		
Unknown	1/89 (1.1)	2/91	0/11 (0)	0/11	0.543
Anthropometry. Mean (SD)					
WHZ	-0.24 (1.9)	15/91	-0.03(1.26)	3/11	0.760*
WAZ	-1.04 (1.6)	6/91	-0.93 (0.84)	2/11	0.847*
HAZ	-1.55(1.5)	15/91	-1.73(2.17)	3/11	0.757*
MUAC	15.3 (1.70)	3/91	13.99 (0.70)	1/11	0.017*
SAM (%)	6/75(8.0)	16/91	0/8 (0)	3/11	0.406

†P values are X^2 p values for differences in proportions between case children and control children unless otherwise specified. *2 sided independent ttest **rank sum test

5.3.5 Controls

55 control households were recruited from randomly generated locations in Blantyre, frequency matched on age to index children from the primary RRTE dataset. Characteristics of the 55 children frequency matched on age and compared to index children from the primary study can be seen in Table 5.5. Median age was 11.5 months (IQR 8.2, 15.4). No children had symptoms of gastroenteritis as this was an exclusion criteria for control households. All age matched children were vaccinated against rotavirus. As with the index children a substantial proportion (26/55(47.3%) had previously attended a health care facility with diarrhoea, with 3/55(5.5%) having previous hospital admissions due to diarrhoeal disease. There were no significant differences in birth weight, prematurity or breastfeeding between control children and index children. Household size, presence of electricity at home, and use of a shared toilet also showed no significant difference across groups.

There were however a few differences between control and index child households. Fewer control households spent over 30 minutes collecting water compared to index child households (3/55 (5.5%), compared to 68/197 (34.5 %) of index children (p<0.001)). In 50/55 (90.1%)of control households at least one person had a regular salary compared to 127/196(65.1%) of index households, p<0.001. The wealth index was significantly higher in control households (mean 2.55 (SD 0.52), compared to 2.37 (SD 0.57) in index households (p=0.04). However half of control households reported problems getting the food they needed (29/55, 52.7%), compared to 60/196 (30.6 %) of index households. The majority of control households were recruited in the second two quarters of the year, in comparison to index children who were fairly evenly distributed throughout the year.

There was however no significant difference in the proportion of households recruited in rotavirus season between the two groups (Table 5.6).

5.3.6 Secondary attack rates

5.3.6.1 Secondary attack rates in household contacts of symptomatic children

705 household members were recruited from 196 households. If no samples were given data were collected on symptoms to define secondary attack rate for disease. 6 household contacts were documented as having unknown symptom status at least once during follow up, and these are excluded from the denominator for clinical secondary attack. 665 individuals from 188 households contributed at least one sample, with a total of 1212 samples collected. Secondary attack rates in household contacts of symptomatic rotavirus cases were very high with 434/665 (65.3%) individuals positive for rotavirus. Attack rates were even higher when a sensitivity analysis was performed using any detectable rotavirus as the definition of a positivity (563/665 (84.7%)) (Table 5.8). Viral loads were low, with median copy numbers of 311 (IQR 89, 2298) for sample one and 306 (IQR 80, 1353) for sample two. There was no clear difference in secondary attack based on age of the household contact.

Clinical secondary attack was much less common with 48/699 (6.9%) household contacts reported symptoms of gastroenteritis at any point during follow up. Of these 47 had samples available for testing and 37 (77.1%) were positive for rotavirus, resulting in a SAR for clinical rotavirus disease of 37/698 (5.3%). Rates of clinical disease were significantly higher in children under 5 years (12/91, 13.2% $p<0.001$).

Table 5.8 Secondary attack rates for rotavirus infection and disease in households exposed to rotavirus positive index children, compared to shedding rates in asymptomatic control households

	Definition of secondary attack								
	Infection						Clinical disease		
	>= 100 copy numbers			Any shedding**			Rotavirus positive clinical disease		
	Study	Control		Study	Control		Study	Control	X^2 P*
Overall	434/665 (65.3%)	40/144 (27.8%)	<0.001 †	563/665 (84.7%)	76/144 (52.8%)	<0.001 †	37/698 (5.3)	0/153 (0.0)	0.004 †
Age stratified (years)									
0-4	57/88 (64.8)	2/10 (20.0)	0.006†	76/88 (86.4)	6/10 (60.0)	0.033 †	12/91 (13.2)	0/11 (0.0)	0.200 †
5-14	127/193 (65.8)	14/48 (29.2)	<0.001 †	170/193 (88.1)	24/48 (50.0)	<0.000 †	4/197 (2.0)	0/53 (0.0)	0.296 †
15-45	240/367 (65.4)	5/20 (27.4)	<0.001 †	302/367 (82.3)	44/84 (52.4)	<0.001 †	20/390 (5.1)	0/87 (0.0)	0.031 †
45+	9/16 (56.3)	1/2 (50.0)	0.867 †	14/16 (87.5)	2/2 (100.0)	0.596 †	1/18 (5.6)	0/2 (0.0)	0.732 †
X^2 P*	0.894	0.838		0.583	0.596		0.001		

*p value for difference in proportion across age categories †p value comparing difference in proportion between cases and controls **any shedding defined as any rotavirus detected on VP6 qRT-PCR without confirmatory NSP3 assay

Of the symptomatic household members 42/48 (87.5%) reported symptoms at their initial interview, the remaining 5 developed symptoms subsequently. Of the 42 that reported symptoms at baseline 4 had symptoms which started before those of the index child, and 4 started the same day. The age and stool test results for these individuals are summarised in Table 5.9. One household member who was symptomatic at baseline had missing data for symptom onset. If these household members are excluded, disease attack rate for rotavirus gastroenteritis was 30/690 (4.3%). The serial interval for clinical disease ranged from 1-8 days with a mean of 3.2 days. Household contacts where symptoms started on or before the onset of symptoms in index children were excluded from estimations of serial interval.

Table 5.9 Summary of household contacts with symptom onset prior to or same day as index child

Symptom start date: household contact	Symptom start date: index child	Age of household Contact (years)	Rotavirus positive
15 Aug 2015	15 Aug 2015	37.11	Yes
29 Aug 2015	29 Aug 2015	1.08	Yes
25 Mar 2015	26 Mar 2015	39.87	No
19 Mar 2016	20 Mar 2016	1.03	Yes
19 Mar 2016	19 Mar 2016	1.19	Yes
25 Mar 2015	26 Mar 2015	33.22	Yes
01 Jan 2016	02 Jan 2016	0.91	No sample
24 Jul 2016	24 Jul 2016	32.14	Yes

5.3.6.2 Rotavirus shedding in control households

There was also a high prevalence of rotavirus shedding in the control households, with 40/144 household members positive for rotavirus (27.8%), significantly less than observed in household contacts of a symptomatic case (434/665, (65.3%)) (Table 5.8). No significant difference was seen in shedding patterns across age groups. There was no clinical gastroenteritis in the control households, as this was an exclusion criterion for recruitment.

5.3.7 Genotyping data

5.3.7.1 Genotypes in index child

Out of 195 samples from index children with available genotyping data, almost a third were G2P[4] (60/195, 30.8%). The next most frequent was G1P[8], at 48/195 (24.6%), followed by G2[P6] (28/195, 14.4%) and G12P[6] (16/195 (8.2%). 16/195 (8.2%) were mixed genotypes, and for three samples a G type was not obtained (3/195 [1.5%]) (Fig 5.3).

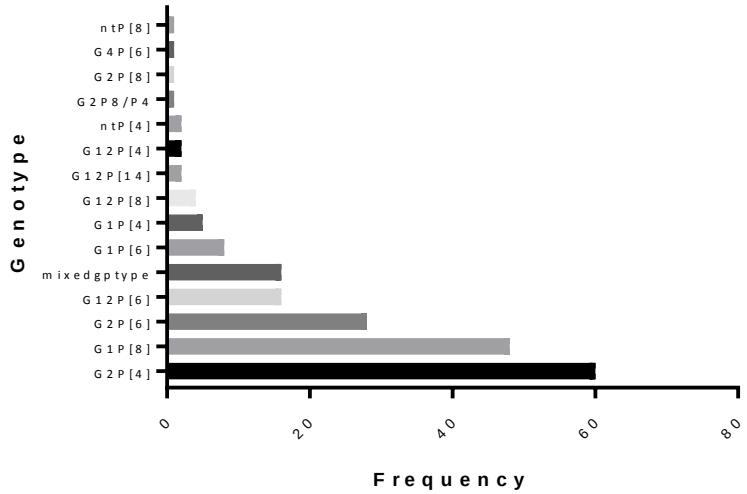


Figure 5.3 Rotavirus genotypes in index children

5.3.7.2 Genotyping in household members

Initially, genotyping was attempted on samples from household members with a Ct value of <38, however very few samples with Ct values between 35 and 38 were successfully typed, so typing was limited to samples with a Ct of ≤35. Only one sample was typed per household contact, if a household member had two samples with Ct values ≤35 the first sample was selected for typing. 297 samples were genotyped from contacts in the 195 households with genotypes available for the index child. Of these, in 94/297 (31.6%) samples the same G and P type was identified in the household contact as identified in the index child. In a further 27/297 (9.1%) and 15/297 (5.0%) respectively the G type or the P type was the same between index child and household contact, but it was not possible to type the second component. In 28/297 (9.4%) samples the G type was consistent between index child and household contact but the P type was different, and in 21/289 (7.1%) the P type was consistent and the G type different. In 44/297 (14.8%) of samples both the G and P types were different between the index child and the household contact. This is summarised in Table 5.10.

Table 5.10. Summary of genotyping results

Number of household contacts	705 from 196 households
Number of samples	1212
Number of contacts with at least one sample	665, from 188 households
Number of samples genotyped	
	195 index children 297 samples from household contacts
Transmission of concordant genotypes from index child to household contact	
Full transmission	94 (31.6%)
G transmission P type not typed	27 (9.1%)
P transmission G type not typed	15 (5.0%)
Neither G or P type typed	22 (7.4%)
Both G & P type different	44 (14.8%)
G transmit P type different	28 (9.4%)
P transmit G type different	21 (7.1%)
G type different, P type not typed	30 (10.1%)
P type different, G type not typed	16 (5.4%)

5.3.8 Decision to stop recruiting

This study was initially powered to investigate differences in secondary attack rates in households were in the index child was vaccinated, compared to those where the index child was not vaccinated. However due to the overwhelming success of the vaccine campaign, extremely rapid uptake of rotavirus vaccine, and higher than anticipated vaccine coverage, this was not possible. In August 2016 only 2 unvaccinated children had been recruited. In a final attempt to recruit unvaccinated children an additional study site was opened where there had been a recent vaccine stock out. After 3 months of recruitment at this site, no additional unvaccinated rotavirus positive cases had been identified and the decision was taken that any additional attempts to recruit unvaccinated children would be futile and recruitment was ceased.

5.3.9 Quality control and validation of cohort

5.3.9.1 Withdrawals

There were 61 households which consented at initial recruitment point but then withdrew from the study. 59 of these completed the initial consent process and index child recruitment form, 2 withdrew before any data collection was conducted. Demographic information was compared between index children who completed the study and withdrawals to look for evidence of systematic differences between groups and evaluate risk of bias. Data for index children and children who withdrew is outlined in Table A4 (Appendix, page 264). Withdrawal children were similar to index children who completed

the study, with no evidence of systematic differences. The only differences observed were in sex, where withdrawal children were significantly more likely to be male (41/59 [69.5%]) compared to index children (108/196 [55.1%], chi squared p 0.049), and in previous presentations with diarrhoeal disease, where withdrawals were more likely to have presented with diarrhoea to a health centre previously (36/59 [61.02) vs 91/196 [46.4%], chi squared p value 0.049. Data from withdrawn households is not used in any of the subsequent analysis.

5.3.9.2 Representativeness of RRTE recruits

Children enrolled into the transmission study were also compared to rotavirus positive, vaccine age eligible children from the diarrhoeal surveillance study who were not recruited into the RRTE study to look for evidence of selection bias in recruits. For the purposes of this analysis, children were defined as rotavirus positive if rotavirus antigen was detected on IC rapid test or EIA. EIA was not performed in real time, so children who were positive on EIA but negative on ICT would not have been identified as eligible for the RRTE study. Sensitivity and specificity of ICT test compared to EIA is described in Table 5.11.

From 16th February 2015 to 11th November 2016 inclusive 287 vaccine-age eligible children were identified as rotavirus positive across all 4 study sites using ICT rapid tests. A further 44 children from QECH were identified as rotavirus positive using EIA (28 of whom had been negative on ICT test, and 16 for whom ICT test was not performed. This gave a total of 331 children positive for rotavirus, 196 (59.2%) of whom were recruited into and completed follow up for the RRTE study. Children enrolled into the RRTE study came from both health centres and QECH, while the surveillance platform only recruited at QECH.

The comparison between groups is outlined in detail in Table A5 (Appendix page 267). Rotavirus positive children from the surveillance study were somewhat younger than children recruited into RRTE (10.2 months, IQR 7.6, 14.9 vs 11.4 months, IQR 8.7, 15.3). Children in the diarrhoeal surveillance platform were less likely to be vaccinated that children in RRTE (8/135 [5.9%] unvaccinated vs 2/196 [1.0%]). Children in the diarrhoeal surveillance platform were more likely to have had previous attendances for diarrhoeal disease (85/135 [62.9%] vs 91/196 [46.4%]. Prevalence of SAM was also higher in the diarrhoeal surveillance platform (27/135, [20.0%] vs 23/193 [11.9%]). Children in the diarrhoeal surveillance platform were more likely to be admitted than children in RRTE

(110/135 [81.5%] vs 111/196 [56.6%]), which likely reflects the different recruitment sites used. There was no significant difference between the two groups in terms of disease severity, prevalence of dehydration, need for IV rehydration, or outcome. There were no significant difference in household characteristics such as electricity at home, sharing a toilet, access to water, water source, challenges obtaining food or in mean wealth indicator.

5.3.9.3 Sensitivity and specificity of IC tests

A summary of samples from the diarrhoeal surveillance study which were tested with both EIA and ICT test can be seen in Table 5.11. 22 samples positive on ICT test were negative on EIA. VP6 qRT-PCR was performed on a subset of 10 of these samples (i.e. those children recruited into RRTE). All were positive for rotavirus on qRT-PCR, with a median Ct value of 24.3, ranging from 17.6 to 37.6. As the decision to conduct qRT-PCR was based on a positive ICT result it is not possible to report qRT-PCR results on EIA positive ICT negative samples. Five index children had no initial bulk stool collected as the initial screening was performed on rectal swab and a bulk stool sample was not obtained at the time. Four of these had a second sample collected. In all four of these the second sample was qRT-PCR positive for rotavirus with Ct values ranging from 15.0 to 26.8. One index child was identified purely on rectal swab with no bulk stool sample. Rotavirus was identified in household contacts, so this child was assumed to be a true rotavirus positive case and included in the analysis.

Table 5.11. Sensitivity and specificity of rapid test, where EIA is taken as the gold standard

		IC test Positive	Negative	Total
Rotavirus EIA	Positive	217	29	246
	Negative	22	450	472
	Total	239	479	718
	Sensitivity IC test	88.2 (83.5, 92.0)		
	Specificity IC test	95.3 (93.0, 97.1)		
	PPV*	90.8 (86.4, 94.1)		
	NPV**	94.0 (91.4, 95.9)		

*Positive predictive value **Negative predictive value

5.3.9.4 Representativeness of household contacts.

Where possible, limited anonymous data were collected on household members who were not recruited, to identify any evidence of systematic bias in the household members who were recruited, and to ensure that household members successfully recruited were

representative. Out of 983 documented members of the 196 households, 901 (91.7%) were recruited, including index children. Data were available for 78 of the 84 refusers. 54/78 (69%) were male. 49/77(63.6%) declined to take part in the study, 23/77(29.8%) were absent from the home during the time frame the study was conducted, and 5/77 (6.5%) gave another reason (not specified) for not taking part. Despite the higher refusal rate in males fathers participated in 137/196 (69.9%) of households. This compares to mothers, who took part in 195/196 (99.49) of households. Fathers who did take part were less likely to contribute a sample compared to other relatives. 15/137 (11%) of fathers contributed no sample, compared to 26/568 (4.4%) of other relatives

5.4 Discussion

In this vaccinated population, very high attack rates (65%) were observed for rotavirus infection in households following contact with a symptomatic index rotavirus case, but a low frequency of rotavirus disease(5%). Frequency of asymptomatic rotavirus shedding was lower in household contacts of asymptomatic control children at 28%. These data confirm the remarkable transmissibility of rotavirus and although the estimate of SAR for rotavirus infection is high, it is consistent with findings from other settings. In New Zealand, 48% of household contacts of a rotavirus index case had rotavirus detected in their stool using EIA(251) which is likely to represent a minimum estimate as EIA is substantially less sensitive than qRT-PCR for detecting rotavirus(341), and in Ecuador SAR determined using qRT-PCR was found to be 55%(106).

In contrast to the high SAR for infection, much lower attack rates were observed for clinical disease (5%) than have been previously observed. In Ecuador disease attack rates were 15%, and in New Zealand 67% for children and 26% for adults(106,251). One possible explanation for the lower disease rates observed in Malawi could be high background force of infection, such that once immunity to clinical disease is obtained it is regularly “boosted”, resulting in a lower risk of symptomatic disease on re-infection. This theory is supported by the high frequency of detectable rotavirus in asymptomatic control household members, suggesting a high frequency of exposure to rotavirus in the community. It is also possible that this finding could reflect under-ascertainment of symptoms. This could occur if symptoms were underreported perhaps because mild symptoms are not considered significant, or because adults or older children may be embarrassed to report symptoms of diarrhoeal disease. In our experience however community members were felt to have a low threshold for reporting symptoms to the

study team because the study team were seen as having the ability to facilitate medical assistance or treatment. Another potential reason for under-ascertainment is that for practical reasons after the initial household visit, symptom data for each household member could be collected from a proxy instead of directly from the household member themselves and it is possible that this was less accurate than obtaining data from each individual household member directly.

It is intriguing that there was no difference in the frequency of rotavirus infection in household contacts with age, either in households exposed to a rotavirus case, or in control households. This is in contrast to findings from the UK, where asymptomatic rotavirus shedding occurred with decreasing frequency with increasing age, and from Ecuador, where secondary attack for infection was higher in children under 10 years compared to those over 10 years(19,106). This finding is particularly interesting given that clinical disease attack rate does depend on age, with a significantly higher risk of disease attack in children under 5 years of age. It is possible that in Malawi, exposure to rotavirus generates protection against clinical disease, but not against infection. This could reflect sub-optimal immune response in Malawi, which would fit with the widespread observation that children demonstrate less robust immune response to rotavirus vaccine in low income compared to high income settings(298), and that children from LIC seem to require a greater number of severe disease episodes to generate protection compared to high income countries(38). It could also reflect differences in contact patterns. Households in LIC such as Malawi often live in much closer proximity to one another than households in high income countries, and children are often cared for by many different family members, so this finding could reflect also a more equal distribution of exposure in Malawi. While serial interval range (1-8) reported in this study is consistent with that from previous studies, the mean is somewhat shorter (3 vs 4 to 7 days(76,251)). This may reflect errors in reporting – literacy levels are low in Malawi and accurate estimations of time can be difficult to obtain, but could also reflect differences in contact intensity or water and sanitation.

Although in approximately one third of genotyped samples the genotype identified was consistent between index house and household contact, there were considerable numbers of households where there were inconsistencies between the genotype in the index child and that observed in contacts. This is consistent with findings from Ecuador(106), and is perhaps unsurprising given the high levels of asymptomatic shedding identified in the community without recent history of exposure to an index child. It does

however highlight the complexity of rotavirus transmission in low income settings. The high background circulation of rotavirus may provide frequent opportunity for viral re-assortment, and may provide one explanation for the wide diversity of genotypes previously observed in rotavirus surveillance in Malawi(114,342–344).

This study also demonstrated a high frequency of detectable rotavirus (28%) in household contacts of asymptomatic control children. This is substantially greater than observed in Ecuador, where 12% of asymptomatic infants had detectable rotavirus in their stool(106), and no household contacts of these infants had detectable rotavirus, and also greater than reported from the UK, where age adjusted prevalence of detectable rotavirus in asymptomatic individuals using qRT-PCR was 11%(19). It is however consistent with studies from Burkina Faso, Tanzania and Malawi were prevalence of rotavirus using qRT-PCR in asymptomatic children ranged from 18-31%(20,107,108). It also corroborates findings from historic studies of asymptomatic infants and adults in south America and Africa. In Mexico 30% of children and 21% of adult contacts were found to be EIA positive for rotavirus, and in Nigeria in 1996 30% of 821 asymptomatic adults and children were found to be EIA positive for rotavirus(105,112). Given the higher sensitivity of qRT-PCR for rotavirus, this is likely to reflect a similar frequency of low level shedding to that observed in this dataset. Although the frequency of rotavirus shedding in the community is high, it seems plausible given what is known about the high burden of rotavirus in Malawi, and levels of poverty with associated issues of crowding and poor water and sanitation.

5.4.1 Limitations

It is possible that the high frequency of rotavirus shedding in households reflects cross contamination between samples. This is unlikely at the laboratory level, as negative controls were included for each stage of the analysis, and any assays which failed were repeated. In addition to this any sample with a Ct value of >35 and or copy numbers of <100 had a repeat qRT-PCR performed using a different target (NSP3), and was reclassified if negative on NSP3. Cross contamination at the household level is difficult to exclude entirely, as samples were, by necessity, collected by the family in the absence of the field team. However, every effort was made to counsel families on how to collect samples appropriately and it seems unlikely that non-compliance would be systematic. Because of unavoidable delays in obtaining relevant permission to recruit control households all control samples were collected in a limited time period between July and January 2016, so one explanation for the high frequency of detection of rotavirus in household contacts

of asymptomatic control children could be seasonal changes in the prevalence of rotavirus, although this is perhaps less likely as historically the rotavirus season in Malawi has been between approximately May to October.

While control households were largely comparable to households of index children control households, the wealth index was significantly higher in control households than in households of index cases. This could represent a degree of selection bias in recruitment of controls – controls were selected using a random walk from a random location and it could be that more easily accessible houses are wealthier. This could also represent random chance. However given the similarities in the majority of the rest of household characteristics this is unlikely to have a substantial impact on rates of transmission. It is interesting that despite the fact that control households were wealthier a larger proportion of control households reported problems getting the food they needed, compared to index households. Again, this may represent random chance, or it could reflect time of recruitment. Food supply varies with season in Malawi and the majority of control households were recruited in the latter two quarters of the year, which is the beginning of what is known at the hungry season. In contrast recruitment of index households was reasonably evenly distributed throughout the year.

Children recruited from health centres were comparable to children recruited from QECH, with the exception of disease severity which was greater at the tertiary centre (QECH). This is expected, as the sickest children are referred to QECH and recruitment at health centres was deliberately expanded to increase recruitment of children with less severe disease. Children also tended to be more wasted at QECH. This could reflect a tendency of less well-nourished children to develop more severe disease(345), or could reflect greater levels of dehydration due to a greater severity of disease. It is interesting that a significantly greater proportion of children presenting to QECH had previous attendances for diarrhoeal disease compared to those presenting to health centres. This could be random error, or reflect recall bias. However it could also reflect a phenomenon observed in India, where it was identified that a subset of children develop repeated episodes of severe disease without mounting protective immunity(346).

Despite careful consent processes, post recruitment withdrawal rates were high(24%). Most recruits completed an initial data collection form before withdrawing so we were able to compare demographics between those who completed the study and those who did not, and investigate for any evidence of selection bias. There were significant

differences in sex and previous presentation with diarrhoeal disease between withdrawals and children completing the study, but this seems likely to be a result of chance rather evidence of systematic differences. There were no other significant demographic differences between withdrawals and children who completed, and it is likely that the children who completed the study are a representative sample of children presenting to health care facilities in Blantyre with diarrhoeal disease.

Although the overall numbers of unvaccinated rotavirus positive children identified were very low (10), children who took part in the RRTE study were significantly less likely to be vaccinated than vaccine age eligible children in the diarrhoeal surveillance platform only. Vaccine coverage in Blantyre is now high enough that unvaccinated children are a biased group, and likely not representative of the population as a whole. There also may be a reluctance for families to consent to taking part in a study about rotavirus vaccine if a child was unvaccinated, and then subsequently required admission for rotavirus disease. There were however no other significant differences between the groups suggestive of systematic bias. In households which did consent to take part in the RRTE study, 91% of eligible household contacts consented to take part in the study. The majority of those who declined to take part were male, which is likely to reflect a combination of males being absent from the home more, and cultural factors surrounding compliance in males and the requirement for stool samples. Despite this, in the majority of households fathers took part in the study (70%) and although fathers were underrepresented in comparison to other household members, there are sufficient numbers of adult males and fathers in the sample that it seems likely that the results for male family members are representative of the population.

ICT rapid tests were used to identify children for recruitment into this study. When eligibility is based on the results of a diagnostic test there is always the risk of misclassification. In the RRTE study however the sensitivity of the IC rapid test was very good compared to an EIA gold standard (88.2%), and there was no false positive case recruited. A small number of potentially eligible children were not identified because of false negative IC test results, however sensitivity analysis has shown no evidence of differences between rotavirus positive children who were recruited and rotavirus positive children who were not recruited, so this is unlikely to have affected the results.

One unavoidable problem with the design of this study is that direction of infection cannot be certain. It would be impossible to define direction of infection clearly without a

prospective cohort study, which was not feasible given available time and resources. Previous studies have shown that in the majority of occasions it is infants which bring rotavirus into the house(245), and the low viral loads and low frequency of clinical disease in household contacts in this study corroborate this. Considerable care was taken in this study to collect symptom data on all household members for the 10 days preceding the date of presentation in the index child. In a small number of households (8), other household members reported onset of symptoms the same day, or the day before symptom onset in the index child. These have been included in the primary analysis given that the numbers are small, the time difference between symptom onset is narrow, and this small number of cases is unlikely to have a significant impact on results.

5.4.2 Implications, conclusions and future work

This study demonstrates a high frequency of rotavirus infection in household contacts of rotavirus positive cases in a vaccinated population, but a low frequency of clinical disease. Rotavirus shedding in household contacts of asymptomatic control children was lower, but still substantial. Programmatic rotavirus vaccination was introduced into Malawi 4 years ago and vaccine coverage is now over 90% in Blantyre, so this study raises questions about the impact of rotavirus vaccine on asymptomatic rotavirus shedding, and the ability of rotavirus vaccine to substantially reduce population level transmission. The role of asymptomatic rotavirus infection in ongoing transmission in the community is as yet unknown, and a topic for future study.

It is important to note that household contacts in this study appear to be protected against rotavirus disease with increasing age, but not against infection, and that protection against disease seems to be more substantial than observed in other countries. If this protection against disease relies on frequent exposure to rotavirus throughout life, then an increase in rotavirus disease in older age groups may be observed as incidence of rotavirus gastroenteritis falls following vaccine introduction. In contrast, household contacts in this study seem to have less immunity to infection than seen in other populations such as the UK, where asymptomatic rotavirus shedding decreases in frequency with increasing age. Both of these observations require further study, and ongoing monitoring to investigate the long term impact of vaccine on these effects.

This study was originally designed to investigate the effect of vaccine exposure on the risk of rotavirus transmission to household contacts of a symptomatic rotavirus index case. Due to the excellent vaccine coverage in Blantyre it is not possible to answer that question

directly, but it may be possible to explore using more complex analytical techniques and mathematical models. In order to do this, outstanding questions on the relationship between disease severity and viral shedding in index children and predictors of rotavirus transmission in households must be answered. These questions will be addressed in the next two chapters.

These are the first data on rotavirus transmission from sub-Saharan Africa, and provide important baseline data from which further studies into risk factors for transmission and potential strategies to reduce transmission can be built. In particular, because of the complex nature of rotavirus epidemiology and immune response to exposure and vaccine, mathematical models have increasingly been used to evaluate the effect of different vaccine strategies on disease burden, and have provided invaluable insights into the presence of indirect effects and other vaccine effects on the epidemiology of rotavirus(118,237,330,347). As observational studies become increasingly difficult to conduct in view of high levels of vaccine coverage across growing proportions of the global population mathematical models are likely to become increasingly crucial in informing global rotavirus vaccine policy. The accuracy of predictions made by mathematical models largely depends on correct parameterisation, particularly of key factors such as SAR and R0, and providing locally accurate baseline data to inform models is therefore essential(348–350).

Chapter 6. Duration and density of rotavirus shedding in children with rotavirus disease and their household contacts

6.1 Introduction

The previous chapter established that rates of rotavirus transmission in households exposed to a symptomatic index child are extremely high. The next chapters will focus on understanding how viral shedding and other risk factors affect transmission. Rotavirus transmission is predominately person to person direct spread through the faecal-oral route. The passage of viral particles shed in the stool of infected individuals is a necessary step for onward transmission(47), but much remains unknown regarding density and duration of shedding in infected individuals and how this relates to disease severity and risk of transmission to close contacts, particularly in low income settings.

Viral particles are shed in the stool when individuals are infected with rotavirus. These were first identified in duodenal sections from children with acute gastroenteritis by Ruth Bishop in the 1970s using electron microscopy(1), and since then a variety of techniques have been used to evaluate the presence of rotavirus in stool samples. These are described in detail in chapter 1, section 1.2, page 30. Initial studies using electron microscopy or immunoassays revealed that rotavirus was shed in the stool of 50% children prior to the onset of symptoms(253), and that shedding continued for several days after symptoms had resolved(351,352). The advent of molecular techniques to detect rotavirus in stool increased the sensitivity of detection of rotavirus, allowed lower viral loads to be detected and demonstrated that viral shedding in children continued for several days longer than had been previously determined using other antigen detection methods(18). Using RT-PCR Richardson et al described extended viral secretion of 25-57 days in 11/57 (30%) children admitted with clinical rotavirus disease in Australia (254).

Development of a semi-quantitative real-time RT-PCR for rotavirus allowed quantitative estimation of viral loads in stool samples(259,353). Using these techniques, Mukhopadhya et al demonstrated shedding of rotavirus for a median of 24 days in children from southern India with rotavirus gastroenteritis and 18 days in children with asymptomatic infection(255). They also demonstrated a rapid decline in viral load after the resolution of symptoms in children with disease, and described lower viral loads in children with asymptomatic infection compared to symptomatic disease. Kang et al showed a strong positive correlation between disease severity defined using the 20 point Vesikari score and viral load, and a positive association between the frequency of passage of diarrhoeal

stools and viral load(259). Phillips et al were able to identify a cut-off in qRT-PCR cycle threshold that correlates with the presence of clinical disease(354) and a similar cut-off has also been identified in children from Malawi(20). The use of qRT-PCR has also identified a high frequency of asymptomatic infection in community members of all ages, but of highest frequency (30%) in young children(19). It is possible that low level asymptomatic shedding plays a substantial role in community transmission of rotavirus infection, but the degree to which this is the case is not well described.

Since rotavirus vaccination mimics natural infection, which provides incremental protection against severe rotavirus gastroenteritis, then it is possible that prior vaccination could mitigate disease severity in the event a vaccinated child develops rotavirus disease. If, as found in India(259), disease severity correlates with faecal viral shedding density, it follows that vaccination may be able to reduce viral shedding, and therefore reduce transmission to close contacts, even in the event of clinical vaccine failure. If this hypothesis is correct, this could be particularly important in low-income high-burden settings, where vaccine effectiveness against symptomatic disease is lower.

Most of the available data, excepting India, come from high income settings. Individuals from low income settings may differ in intestinal integrity, nutritional state, immune response or co-morbidities, and this may lead to different shedding patterns compared to high income settings. For example, Cunliffe et al in Malawi identified that HIV-infected children with rotavirus gastroenteritis continue to shed for longer following an episode of rotavirus gastroenteritis compared to children who were HIV negative(260).

Improved understanding of vaccine impact on rotavirus transmission requires a better defined relationship between the symptom severity and viral shedding in low income settings. This study therefore aimed to describe patterns of rotavirus shedding over time in infants with rotavirus gastroenteritis and their household contacts and to investigate factors associated with faecal viral load in vaccine age eligible children with symptomatic disease in Malawi.

6.2 Methods

6.2.1 Objectives

1. To describe change in rotavirus viral load over time from symptom onset in symptomatic index children and in household contacts
2. To identify factors associated with viral shedding density in symptomatic index children
3. To describe duration of rotavirus shedding in symptomatic index children and in household contacts of index children who are found to be shedding rotavirus

6.2.2 Study design

These data come from the prospective cohort study of children with clinical rotavirus disease and their household contacts, described in detail in Chapters 2 and 5, and from a smaller cohort nested within the primary study of 21 index children and their household contacts in whom more intensive sampling was carried out for a longer period of time.

6.2.3 Study site

Participants for this study were recruited from QECH, Zingwangwa Health Centre, Gateway Health Centre and Madziabango Health Centre.

6.2.4 Study population

Study population are described in detail in Chapter 5. Additionally, the nested intensive cohort comprised 21 children and their household contacts in whom more detailed 28-day follow up was conducted.

6.2.5 Study Procedures

6.2.5.1 Enrolment

Enrolment into the primary study is described in Chapter 5 (section 5.2.7, page 155). Enrolment procedures and data collection for the nested cohort were the same, with the exception that recruitment took place from 16th Feb to 14th April 2016 and follow up occurred for a longer time period. Eligibility criteria for index children and their household contacts were identical to those used in the primary study and described in Chapter 5 (Sections 5.2.7.4, page 157 and 5.2.7.8, page 160).

6.2.5.2 Sample collection

Primary study:

Each index child had two bulk stool samples collected (Fig. 6.1):

- Sample 1: at presentation to health care facility and recruitment into the study
- Sample 2: 5 to 7 days after symptom onset

Each household contact had two stool samples collected

- Sample 1: days 5 to 7 after the onset of symptoms in the index child
- Sample 2: days 10 to 12 after the onset of symptoms in the index child

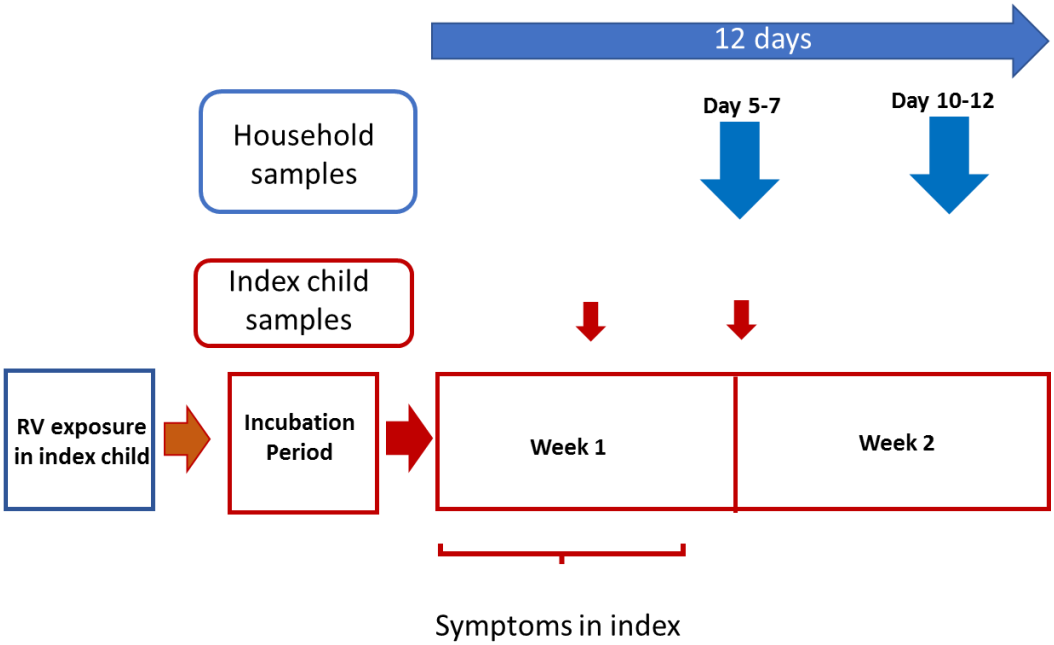


Figure 6.1 Sample collection in primary study

Nested cohort

Index children had up to 11 stool samples collected (Fig. 6.2):

- daily from time of presentation for the first 7 days after symptom onset
- twice weekly from 7 until 14 days after symptom onset
- weekly from day 14 until day 28 after symptom onset

Household contacts had up to 4 stool samples collected:

- days 7, 14, 21 and 28 after symptom onset in the index child

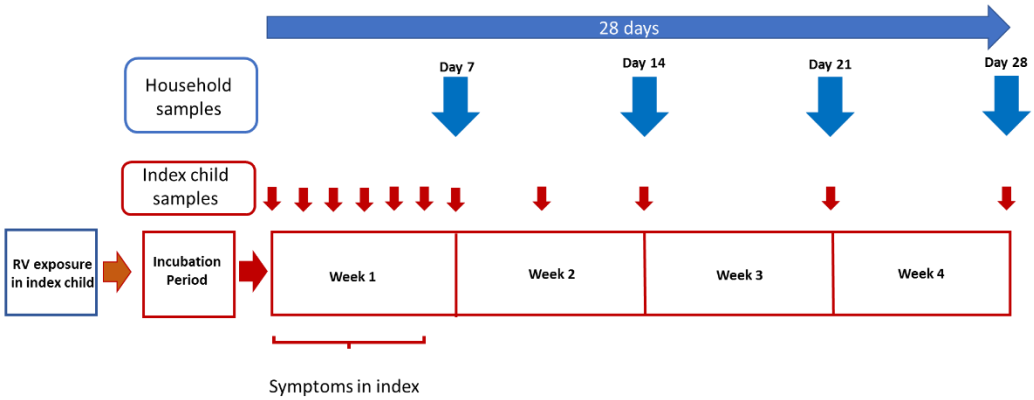


Figure 6.2 Sample collection in nested cohort

6.2.5.3 Household visits and follow up

Household visits were conducted according to the schedule described above. Procedures and data collection were as described in Chapter 5, section 5.2.7, page 155. Data on symptoms in both the index child, and their household contacts were collected at the same time as each sample was collected.

6.2.6 Sample size calculation

No formal sample size calculation was conducted for the exploratory nested cohort. 20 households were selected as the limit of what was practical and feasible in the contexts of the primary study. Because of difficulties in follow up in one of the households an additional household was recruited.

6.2.7 Laboratory procedures

Laboratory procedures were described in detail in Chapter 2, section 2.3, page 87 and chapter 5, section 5.2.7, page 155. Stool samples from all recruits were tested using real-time qRT-PCR (RT qRT-PCR) to assess stool viral load.

6.2.8 Statistical analysis

Rotavirus shedding was defined as the presence of 100 or greater viral copy numbers in stool on VP6 qRT-PCR and positive on confirmatory NSP3 assay if Ct value was ≥35, as described in chapter 5, section 5.2.13, page 168. The exception to this was for analysis of changes in viral load over time in index children and in rotavirus positive household contacts where all data were included regardless of viral load.

Faecal viral load distributions were plotted, and central tendency described using median and interquartile range [IQR]. Viral load did not follow a normal distribution so was log-

transformed for further analysis using the natural logarithm. Change in viral load over time and relationship between symptoms and viral load were evaluated using linear mixed models with a random intercept to account for the within child clustering resulting from repeated measures. To account for the non-linear relationship between faecal viral load and time, polynomial terms (quadratic and cubic) were included in the model for viral load over time in index children. These were selected based on the best visual fit to the data. Polynomial terms did not improve the visual fit in the models for viral load over time in household contacts so were not included.

Relationship between faecal viral shedding load and disease severity was investigated using linear regression. The outcome variable was peak log-viral load in index children. Variables achieving a Z test p value of ≤0.1 on univariate analysis were selected for evaluation in the multivariable model. Age and sex were included *a priori*. Nested models were compared using F tests. Only one variable of a set of collinear variables (such as diarrhoeal duration and diarrhoeal frequency) was selected for inclusion in the final model. 3 outlying values with high statistical leverage were excluded from the final model.

Time-to-event analysis was used to describe the duration of shedding in index children and in household contacts, where the event of interest was defined as cessation of shedding. Cessation of shedding was defined as the first time point from which no rotavirus was subsequently detected until censoring. Thus, an individual with no detectable rotavirus at a given analytical timepoint but who was shedding rotavirus in subsequent samples, was classified as having ongoing shedding at the timepoint of analysis. Sampling duration was limited to 28 days. For those who ceased shedding it was assumed that there was no viral shedding beyond truncation. For index children the start time for analysis was the onset of symptoms, for household contacts start time was defined as the first positive sample.

6.3 Results

This analysis was conducted in 4 parts.

i) Rotavirus positive children with gastroenteritis from the primary study.

These were children with symptomatic rotavirus disease from the primary database who had two stool samples collected in the first week after presentation. This dataset was used to analyse associations with viral shedding density

ii) Rotavirus positive children with gastroenteritis from the nested cohort.

These were the 21 children with up to 11 samples collected over a 28 day period following symptom onset. Data were used to investigate trends in faecal shedding over time and duration of shedding in symptomatic children

iii) Household contacts of symptomatic index children from the primary study

This comprises the primary dataset in which two samples were collected in the 12 day time period after the onset of symptoms in the index case and was used to investigate temporal relationship between faecal viral load in contacts and symptom onset in index children.

iv) Household contacts of the 21 children recruited into the nested cohort

These household members had two additional samples were collected at 21 and 28 days after symptom onset. These data were used to describe duration of shedding in household contacts.

6.3.1 Rotavirus positive children – primary dataset

374 samples were collected in total; 189 first samples and 185 second samples (5 children had rectal swabs alone collected at the time of first testing, and two bulk samples were lost during processing).

Samples 1 and 2 were collected respectively a median of 3 (IQR 2,4) days and 5 (IQR 3,7) days after symptom onset. Sample 1 median viral loads were significantly higher, with median Ct value of 19.1 (IQR 17.2, 22.2) corresponding to median copy numbers of 1.67×10^7 (IQR 1.63×10^6, 6.37×10^7) in Sample 1, compared with median Ct value of 22.61 (IQR 19.04, 29.14) corresponding to median copy numbers of 5.5×10^5 (IQR 7.9×10^3, 7.31×10^6) in Sample 2, sign rank p value <0.001.

6.3.1.1 Predictors of viral load

On univariate analysis a significant positive association was demonstrated between peak viral load and clinical disease severity as measured by the standard 20-point Vesikari score (see Chapter 5, section 5.2.7.5, page 158) (Table 6.2, Fig. 6.3, regression coefficient 0.24[95% CI 0.10, 0.38]). The presence of vomiting (regression coefficient 1.80 [95% CI 0.32, 3.28] and admission to hospital (regression coefficient 0.98 [95% CI 0.21, 1.76]) were also strongly associated with viral load. Weak evidence of a positive association with diarrhoeal duration, vomiting duration of at least 3 days, and severe dehydration were also identified. In addition peak viral load was significantly positively associated with birth

weight and height for age Z score (HAZ) and negatively associated with weight for height Z score (WHZ) and there was weak evidence of a negative association with mid-upper arm circumference (MUAC) (Table 6.1).

On multivariable analysis a positive association with Vesikari score (regression coefficient 0.17 [95% CI 0.04, 0.312]) and a negative association with WHZ (regression co-efficient -0.26 [95% CI -0.46, -0.02]) were retained. Sex and age were included *a priori*. There was weak evidence that including diarrhoeal duration improved the model (F test p value 0.0672) in addition to Vesikari score, but this was not included in the final model due to concerns around collinearity.

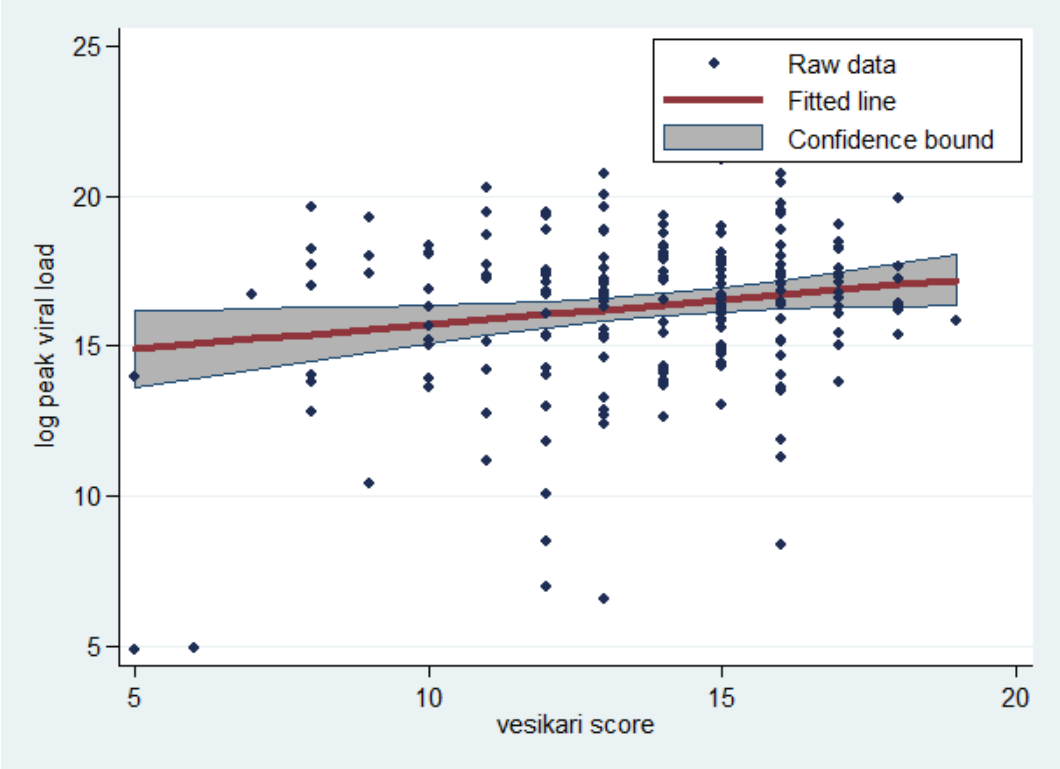


Figure 6.3. Relationship between log peak viral load and Vesikari score. Raw data are log(peak viral load), and the fitted line represents the regression line. Confidence bound represents the 95% confidence limit either side of the fitted valued. Regression coefficients can be seen in Table 6.1

Table 6.1. Univariate and multivariate analysis of potential predictive factors for peak viral load in index children

Covariate	N	Univariate association with peak viral load	P value*	Multivariate association with peak viral load	P value*
Sex (Male)	195	-0.56 (-1.34, 0.22)	0.157	-0.60, (-1.33, 0.14)	0.110
Age in months	195	-0.04 (-0.10, 0.02)	0.245	0.00 (-0.06, 0.06)	0.964
HIV exposed	195	0.13 (--1.03, 1.29)	0.823		
HIV infected	58	0.58 (-3.55, 4.71)	0.779		
Premature	195	0.34 (-1.75, 2.43)	0.748		
Birth weight (kgs)	195	0.23 (0.01, 0.44)	0.038		
Ever breast fed	195				
Yes		-1.18 (-6.62, 4.25)	0.668		
SAM					
Yes	193	0.17 (-1.04, 1.38)	0.780		
WHZ	194	-0.39 (-0.63, -0.15)	0.001	-0.26 (-0.46, -0.02)	0.032
WAZ	194	0.10 (-0.24, 0.43)	0.565		
HAZ	190	0.28(0.12, 0.43)	0.001		
MUAC (Cm)	194	-0.26 (-0.56, 0.04)	0.090		
Diarrhoea episodes**	195				
1-3		REF			
4-5		0.60 (-0.67, 1.87)	0.350		
≥6		0.69 (-0.58, 1.96)	0.286		
Diarrhoea duration (days)	195				
1-4		REF			
5		1.60 (-0.00, 3.21)	0.051		
≥6		-0.44 (-2.11,1.23)	0.604		
Vomiting	195				
Yes		1.80 (0.32, 3.28)	0.018		
Vomiting frequency	181				
<5		REF			
≥5		0.06 (-0.78, 0.88)	0.892		
Vomiting duration (days)	181				
1		REF			
2		0.62 (-0.64, 1.88)	0.333		
≥6		1.33 (0.15, 2.52)	0.028		
Dehydration	195				
None		REF			
Some		0.65 (-0.53, 1.83)	0.282		
Severe		1.31 (-0.03, 2.65)	0.055		
IV fluids	195				
Yes		0.57 (-0.28, 1.42)	0.185		
Oral fluids	195				
Yes		0.24 (-1.44, 1.92)	0. 775		
Admission	195				
Yes		0.98 (0.21, 1.76)	0.013		
Outcome	195				
Home		REF			
Died		-2.03 (-5.88, 1.80)	0.297		
Vesikari score	192	0.24 (0.10, 0.38)	0.001	0.17 (0.04, 0.312)	0.013

6.3.2 Rotavirus positive index children from nested cohort

178 samples were collected from 21 children over a period of 29 days from symptom onset. Viral load declined significantly over time since symptom onset (Fig 6.4) with a regression coefficient for relationship between log copy numbers and time in days since symptom onset of -1.60 (-2.44, -0.74, p<0.001) (Table 6.3). Viral load was significantly higher when children were symptomatic, regression coefficient (6.44, 95% CI 4.63, 8.25) (Fig 6.5). When adjusted for time since onset of symptoms the trend toward higher viral loads persisted but was no longer significant at the 5% level (regression co-efficient 1.45, 95% CI -0.27, 3.17).

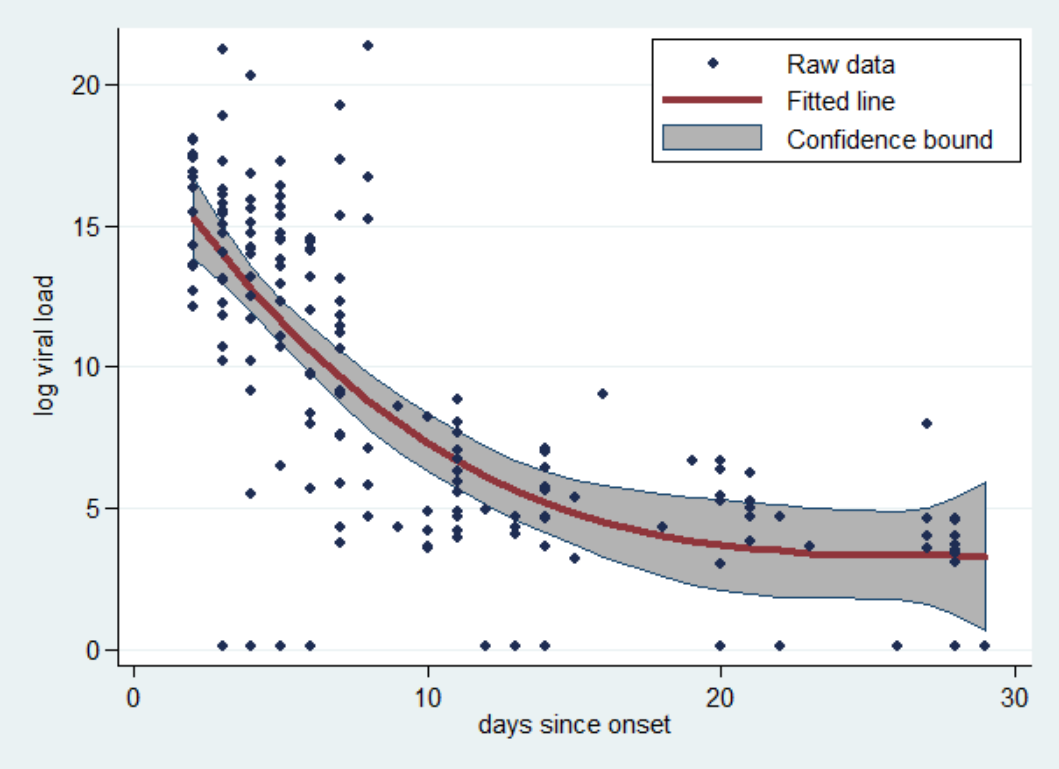


Figure 6.4. Decline in viral load over time in symptomatic children. Raw data is log(viral load), and the fitted line represents the regression line including quadratic terms to account for the non-linear nature of viral decay. Confidence bound represented the 95% confidence limit either side of the fitted valued. Regression coefficients can be seen in Table 6.2

Table 6.2 Regression model for shedding curve

Log viral load	Regression coefficient	P value	95% Confidence limits
Time since symptom onset (TS)	-1.60	<0.001	-2.44, -0.74
$(TS)^2$	0.06	0.110	-0.01, 0.13
$(TS)^3$	-0.01	0.403	-0.00, 0.00

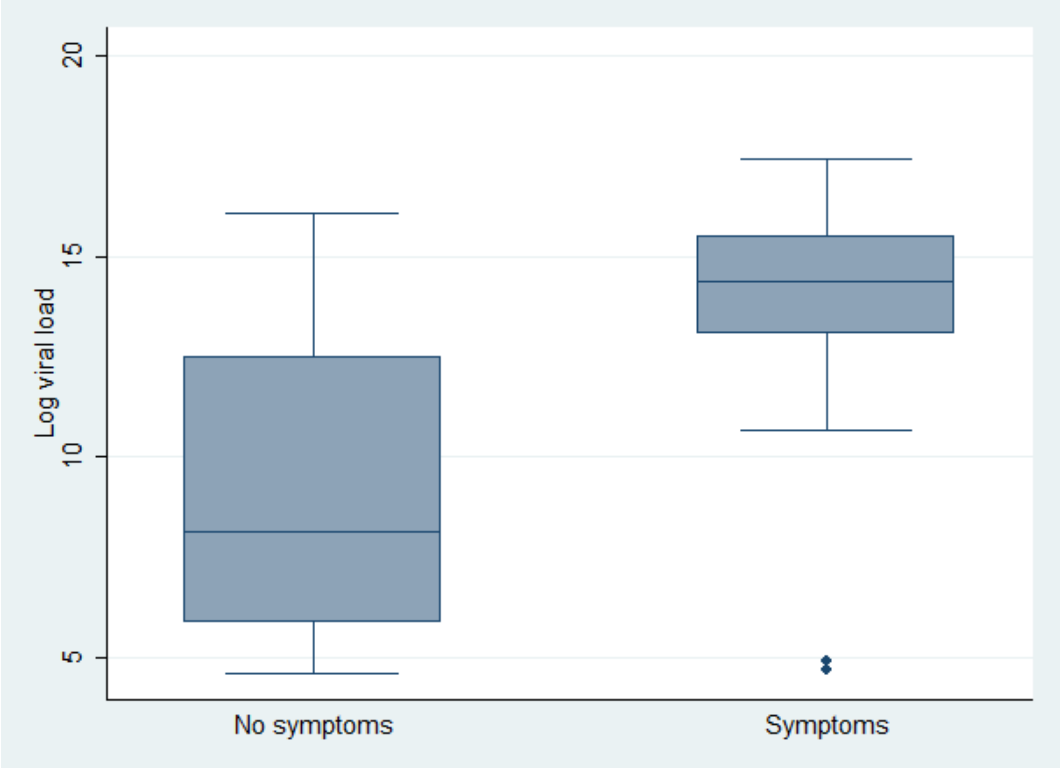


Figure 6.5 Log viral load by presence or absence of symptoms. Error bars represent mean and standard deviation.

The proportion of children shedding rotavirus declined significantly with each visit, from 100% at the first visit to 20% at the final visit (Table 6.3, Fig 6.6). 7 children were still shedding rotavirus at the time of their last follow up visit. Of those who did stop shedding whilst in follow up median duration of shedding was 27 days (IQR 19, 28)

Table 6.3. Proportion of children shedding rotavirus at each visit

	Visit number										
Shedding	1	2	3	4	5	6	7	8	9	10	11
No	0(0)	3 (14)	4 (14)	2 (10)	3 (15)	6 (32)	7 (41)	3 (23)	6(55)	5(56)	4(80)
Yes	21(100)	18(86)	16(80)	19(90)	17(85)	13(68)	10(59)	10(77)	5(45)	4(44)	1(20)
Total	21	21	20	21	20	19	17	13	11	9	5

Chi squared p value<0.001. Numbers in brackets are percentages

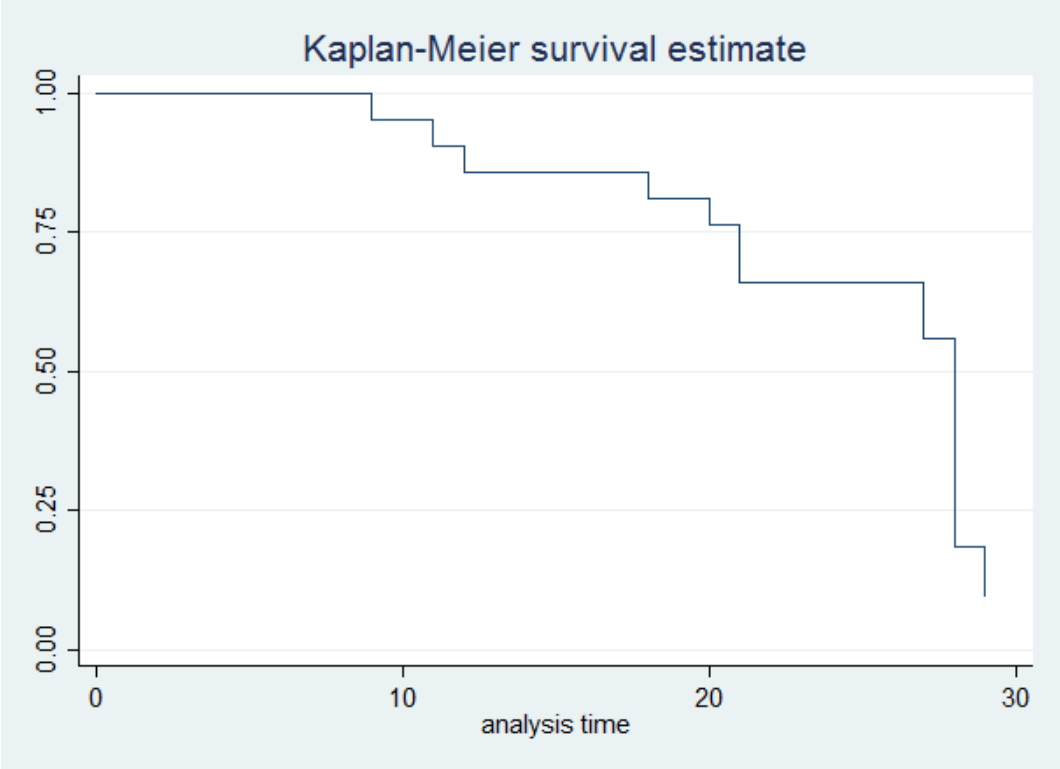


Figure 6.6. Kaplan Meier curve of time to cessation of shedding in index children. Analysis time is in days since symptom onset

6.3.3 Household contacts of symptomatic children

1312 samples were collected from 665 household contacts. 1212 of these were from the 606 individuals in the "primary study" with 2 samples collected over 2 weeks from onset of symptoms in the index case and 100 were from the 59 individuals in the intensive cohort with up to 4 samples collected over 28 days. Data from both groups were analysed together to examine changes in viral load over time. Viral loads were considerably lower than those observed in children presenting with rotavirus disease, with a median Ct value across all the samples of 34.8 (IQR 31.8, 36.6), corresponding to a median viral load of 712 (IQR 256, 3704). Timing of samples in days since symptom onset in index child can be seen in Table 6.4.

Table 6.4 Timing of sample collection household contacts in relation to symptom onset in the index child

	Median time in days since symptom onset in index child (range).
Sample 1	5 (1, 18)
Sample 2	11 (7, 39)
Sample 3	21 (19, 32)
Sample 4	28 (26, 40)

Viral load in household contacts declined significantly over time from symptom onset in the index child (Fig. 6.7), with a regression coefficient for relationship between log copy numbers and time since symptom onset in index child of -0.05 (95% CI -0.02, -0.08) (Table 6.5).

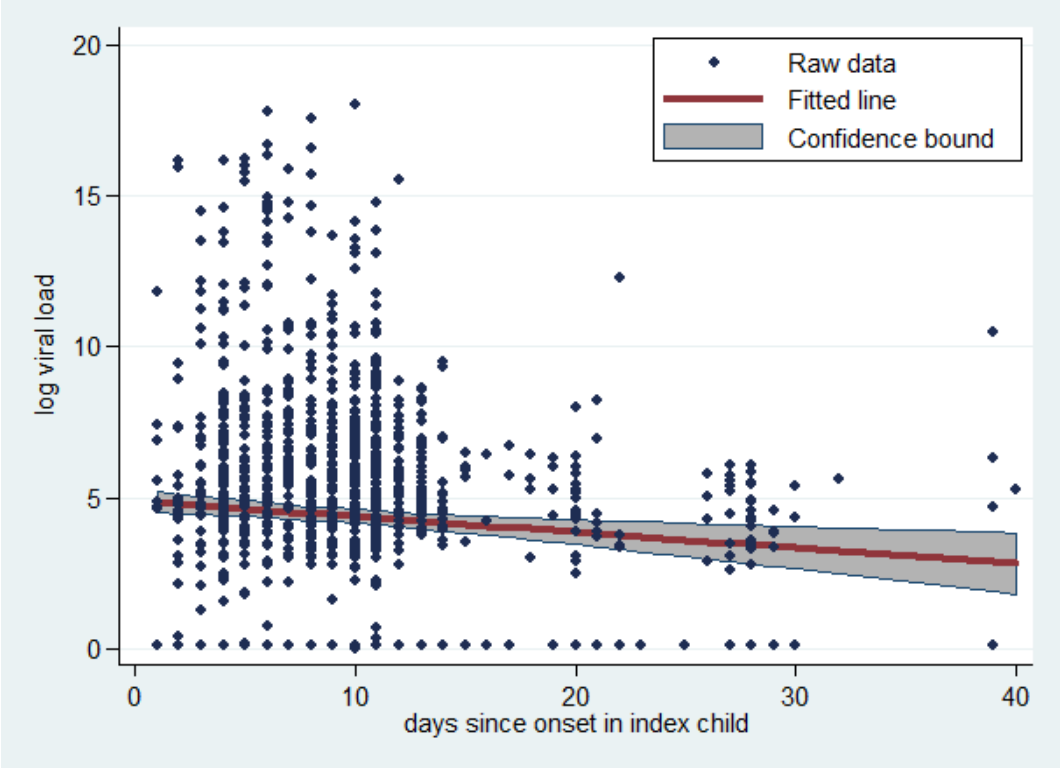


Figure 6.7 Viral load in household contacts since time of symptom onset in index child. Raw data is log(viral load), and the fitted line represents the regression line. Confidence bound represented the 95% confidence limit either side of the fitted valued. Regression coefficients can be seen in Table 6.5

Table 6.5 Regression model for log viral copy numbers in household contacts since symptom onset in the index child

	Coef.	P value	95% CI
Time since symptom onset in index child (TSI)	-0.05	0.001	-0.08, -0.02

Log viral load was significantly higher in those household members with symptoms of gastroenteritis (Fig 6.8) , with a regression coefficient for relationship between log copy numbers and presence of symptoms in the household contact of 3.36 (95% CI 2.33, 4.40, p<0.001)

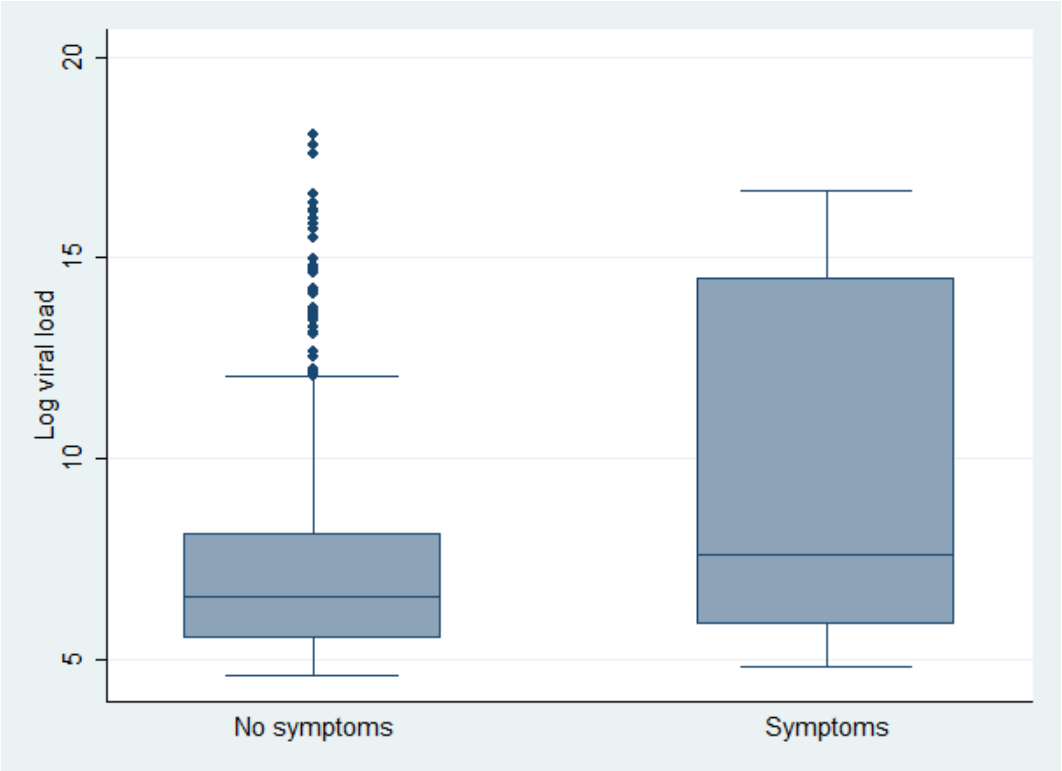


Figure 6.8 Log viral load in household contacts by presence or absence of symptoms. Error bars represent mean and standard deviation.

6.3.4 Household contacts of children in nested cohort

In the 59 household contacts of children in the nested cohort the proportion of household members shedding rotavirus declined significantly over time, from 76.3% at visit 1 to 31.82% at visit 4, chi squared p value 0.001 (Table 6.6). Time to cessation of shedding can be seen in Fig. 6.9. For this analysis the start of shedding was defined as the first positive sample. 29/59 (49.2%) individuals stopped shedding rotavirus before follow up was completed. For these individuals median time to cessation of shedding was 19 days (IQR 17, 23), with a range of 9-26 days. 16/59 (27.1%) individuals continued to shed rotavirus

throughout follow up, median follow up for these individuals was 23 days (IQR 20.5, 24.5, range 20, 36). This compares to 290/606 (47.9%) individuals in the primary cohort who were still shedding rotavirus at the time follow up ceased. 14/69 (20.2%) did not shed rotavirus at any point during follow up. No new episodes of shedding were identified in week 3 or 4 or follow up - all individuals who had a positive sample at visit 3 or 4 had had a previous positive sample at visit one or two. Only two contacts in the nested cohort developed symptoms, and there was no significant difference in median time to cessation of shedding compared to those with asymptomatic infection, so they were included in the analysis.

Table 6.6. Proportion of household members shedding rotavirus by at each visit

	Visit			
Shedding	1	2	3	4
No	14	12	34	30
Yes	45 (76.3)	40 (76.9)	22 (39.3)	14 (31.82)
Total	59	52	56	44

Numbers in brackets are percentages

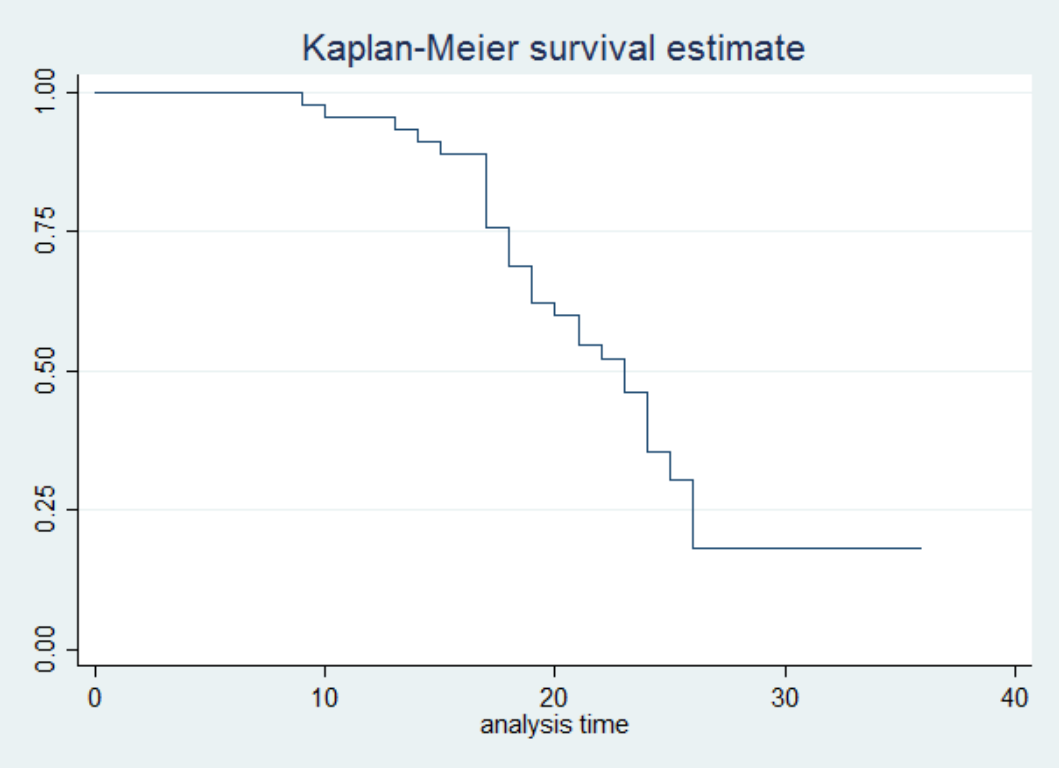


Figure 6.9. Kaplan Meier curve of time to cessation of shedding in household contacts. Analysis time is in days from first positive sample

6.4 Discussion

This study corroborates findings from other settings regarding the relationship between viral shedding density and symptomatic disease. Malawian children with rotavirus gastroenteritis shed rotavirus in high density at the time of initial symptom onset, and continue to shed rotavirus for an extended period of time after symptoms resolve. Disease severity was associated with higher viral shedding density, and symptoms were associated with higher viral loads than asymptomatic infection.

The pattern of rotavirus shedding observed in symptomatic rotavirus cases is similar to that observed by Mukhopadya et al in children from India(255). Initial extremely high viral titres showed a rapid decline over the first 10 days after symptom onset, and then plateau, with a median duration of shedding of approximately 4 weeks (27 days in Malawi, 24 days in children from Vellore). This is substantially longer than the median duration of shedding of 10 days observed by Richardson et al in Australia(254). This could at least in part reflect differences in sensitivity of the assay used; the same assay was used in India and Malawi, and a different, less sensitive assay used in Australia. However there is good evidence that the immune response to both natural rotavirus infection and to rotavirus vaccine is reduced in low income compared to higher income settings(38,62,95)(96,298) and it is possible the extended duration of shedding observed in this study and in India represents another manifestation of this, reflecting delayed clearance of replicating virus as a result of sub-optimal mucosal immunity.

In household contacts the significant decline of viral load over time since onset of symptoms in the index child and the lack of new infections identified beyond the second week of follow up in the nested cohort supports the hypothesis that the symptomatic index child is the primary source of transmission. Median duration of shedding in household contacts who stopped shedding was similar to that observed in asymptomatic Indian children (19 vs 18 days). Notably 27% of household contacts were still shedding rotavirus at the cessation of follow up, with a median duration of follow up 23 days. This raises the possibility that the true duration of shedding in Malawi is longer than observed in this study. Unfortunately this cannot be confirmed because follow up in this study was truncated at 28 days.

The presence of symptoms was significantly associated with higher viral loads in both index children and their household contacts. This corroborates data from India, Malawi and the UK which found significant differences in viral load between individuals with

rotavirus disease compared to those with asymptomatic rotavirus infection(20,255,354). Disease severity defined using the Vesikari score showed a significant positive association with viral load, in line with findings from Vellore(259). When individual components of the Vesikari score were investigated, the presence of vomiting, duration of vomiting, duration of diarrhoea, presence of dehydration and need for admission showed some evidence of a positive association with viral load. Only duration of diarrhoea was retained in the model once adjusted for Vesikari score, with weak evidence of an additional association with increasing viral load (F test p 0.07). This was not included in the final model because of concerns regarding collinearity with Vesikari score. In India, viral shedding density was associated with diarrhoea frequency, and it was postulated that high viral load could contribute to high diarrhoeal frequency, however we did not find a univariate association between diarrhoeal frequency and viral load. This may reflect differences in data collection practices; in India data on stool frequency was recorded by nurses on the ward but in Malawi we relied on maternal report which could be less accurate. Counting diarrhoeal frequency accurately is particularly difficult in Malawi as children typically rely on cloth wrappers rather than nappies, and admitted children with diarrhoeal disease are often nursed with multiple children in one bed. It may be that more absolute variables such as the presence or absence of vomiting or the need for admission are more reliable proxies for disease severity in very low-income countries such as Malawi.

Viral shedding is negatively associated with WHZ score in our dataset, implying that children shed more virus with increasing levels of malnutrition. As a note of caution in interpreting this, accurate measurement of height in young children is challenging and more error prone than measuring weight or MUAC. The standard deviation for our estimates of all our anthropometrical measures is outside than the range given by the WHO for data quality assessment purposes (Table A6, Appendix page 269)(355), and the point estimate for HAZ for our study is substantially higher than that obtained in the 2010 DHS for Blantyre district (-0.04 vs -1.6(356)), meaning that children in our cohort are taller than expected. This is also reflected in the WHZ measurements which are considerably lower in the RRTE cohort than in the 2010 DHS (-0.59 vs 0.4), Table A3. As a result, our findings on nutritional status should be interpreted with caution, and, whilst acknowledging that children attending healthcare facilities with severe gastroenteritis are a different population from children in the community, our estimates of under-nutrition based on WHZ are likely to be an over-estimate. This likely goes someway to explaining

the counter-intuitive findings between the relationship between WHZ and HAZ on viral load in this study, where increasing WHZ is negatively associated with viral shedding density but HAZ is positively associated with viral shedding density – this likely reflects the relationship between WHZ and HAZ. Despite this, the negative association with WHZ and viral load is corroborated by weak evidence of a negative association between MUAC and viral load, and it is biologically plausible that children with poorer nutritional states could shed more rotavirus due to changes in intestinal mucosal integrity, ability to amount mucosal immunity, and intestinal microbiome and an increased tendency to more severe disease associated with malnutrition(345,357–359). Given the prevalence of undernutrition in LIC, these findings certainly merit further investigation.

6.4.1 Limitations

Unfortunately for logistical reasons our follow up was limited to 2 weeks in the majority of household contacts, and to 28 days in a subset of 59 individuals, and so a substantial proportion of household contacts were still shedding rotavirus when follow up ceased (48% of the 606 of individuals in the main cohort and 27% of the 59 in the extended follow up cohort). Low level shedding in infected individuals can be transient(254), so we defined cessation of shedding as the absence of shedding in current and subsequent samples. If a participant was negative for rotavirus at day 8, but then shed rotavirus again at day 10, this would be classified as ongoing shedding until day 10. Individuals who were not shedding at the time the last sample was collected were assumed to have stopped shedding. This will have resulted in misclassification of some ongoing shedders, and our description of the duration of shedding should therefore be considered a minimum estimate. We were also only able to collect one sample per week from household contacts, limiting the accuracy of the duration of shedding. Whilst the absolute duration of shedding should be interpreted with caution, we can be confident that once infected with rotavirus individuals in Malawi appear to shed virus for an extended period of time. This, combined with the high SAR for rotavirus infections in households, and high rotavirus force of infection in Malawi, may provide an explanation for the high frequency of asymptomatic shedding in community households described in the previous chapter.

6.4.2 Conclusions, implications and further studies

The positive association of viral shedding density and rotavirus severity is important as it suggests that reducing disease severity, for example through vaccination, has potential to reduce viral shedding density and therefore rotavirus transmission. The vast majority of the children in this study were vaccinated so it is clear that vaccinated children in low income settings still develop severe disease, however a reduction in disease severity at the population level may still have potential to contribute substantially to an overall reduction in community transmission.

Children in Malawi shed large quantities of rotavirus following an episode of rotavirus diarrhoea and shed for an extended period of time. A large proportion of household contacts of a symptomatic rotavirus case shed rotavirus, some for several weeks after exposure took place. In combination, this is likely to be a major contributor to the large burden of asymptomatic rotavirus observed in the community. It remains to be seen what effect a sustained rotavirus vaccine programme will have on this substantial pool of asymptomatic infection, and indeed the role of this asymptomatic infection in propagating ongoing transmission remains unknown.

This study highlights several questions for further study. In order to accurately define duration of shedding, a cohort study with extended follow up is required, both for symptomatic children and their asymptomatically infected contacts. Unfortunately such studies can be expensive and logistically challenging. Similar studies on duration of shedding in high income settings using similar molecular assays to detect rotavirus would help identify if there if truly is a difference in shedding duration between high and LIC. The intriguing finding of a potential association between WHZ and viral shedding density merits further investigation in different datasets. Finally, having identified a positive relationship between disease severity and viral shedding density the relationship between these two factors and rotavirus transmission needs further study. This will be explored in the following chapter.

Chapter 7. Risk factors for rotavirus transmission in household contacts of children with rotavirus disease in Blantyre, Malawi

7.1 Introduction

In the context of lower vaccine effectiveness in high burden low-income settings, any vaccine-associated reduction in community transmission of rotavirus may be an important factor in overall disease reduction, both for unvaccinated individuals, and as an additional benefit to those vaccinated. Such additional transmission-mediated effects are collectively termed indirect effects of the vaccine, and have the potential to contribute substantially to population level vaccine impact (Chapter 4, section 4.1, page 125)(215). Understanding what influences rotavirus transmission is important for understanding both the mechanisms underlying and the extent of vaccine mediated indirect effects. Possible mechanisms for indirect effects are outlined in Chapter 1 (section 1.6, page 63), but may include overall reduction in frequency of rotavirus disease in the community such that a susceptible individual is less likely to become exposed; a reduction in the number of susceptible contacts infected by one symptomatic individual (reduction in infectiousness of an index case); or transmission of vaccine virus from vaccinated infants to contacts, such that the contacts also generate immunity (Fig 7.1). The first two mechanisms are termed herd protection, the latter herd immunity(360).

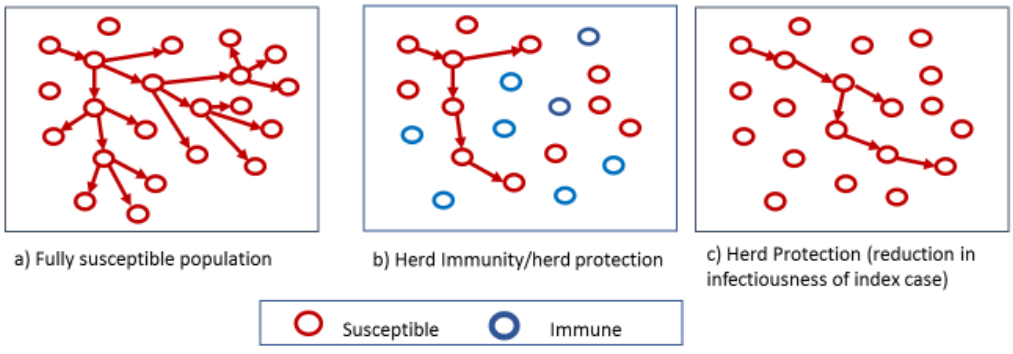


Figure 7.1 Possible mechanisms of vaccine indirect effects

Given that vaccination mimics the incremental protection obtained from consecutive natural rotavirus infections(38,62,95), and that disease severity is positively associated with viral shedding density(8, chapter 6, section 3.6.1.1,page 196), it follows that rotavirus vaccine may have potential to reduce disease severity, viral shedding density, and

therefore transmission to close contacts, even in the event of clinical vaccine failure. Such vaccine effectiveness for infectiousness (rather than disease) has been described with other pathogens such as pertussis(332), but not specifically with rotavirus. In Malawi, where vaccine effectiveness for hospitalised gastroenteritis is approximately 60%, vaccine mediated reduction in infectiousness has the potential to contribute substantially to overall reduction in burden of disease.

To determine vaccine effects on infectiousness and household level transmission it is necessary to determine risk factors for transmission of rotavirus infection and disease within households. Risk factors for transmission can be broadly divided into susceptibility factors or infectiousness factors. Susceptibility factors relate to the relative susceptibility of household contacts to becoming infected and can be divided into population level factors such as climate, household level factors such as relative poverty, and individual factors such as HIV status of the household contact. Infectiousness factors relate to the index child, and examples include disease severity or viral shedding density in the index child.

The existing data on risk factors for rotavirus transmission are limited. There is evidence from the USA that transmission of rotavirus within the household is propagated by the presence of young children(245). At a population level high population density in Dhaka, Bangladesh(270) and flooding(271) in the Solomon Islands have been associated with increased frequency of rotavirus transmission. In terms of household level susceptibility factors household crowding was associated with increased transmission in St Lucia(269). Individual level susceptibility factors associated with transmission of infection and disease were identified in a household study in Ecuador by Lopman et al(106). They identified that being younger than 10 years of age and sharing a room with the index child was associated with susceptibility to rotavirus disease, and being a sibling of the index child was associated with susceptibility to rotavirus infection. The same study investigated infectiousness risk factors for transmission and found that younger age of the index child, increased frequency of vomiting, severe disease, and higher faecal viral load was associated with risk of transmission of disease, and vomiting and disease severity were weakly associated with risk of transmission of infection.

Both Ecuador and St Lucia are upper middle income countries, and to date there are no detailed data on rotavirus transmission from LIC where population structures, contact patterns, living conditions, comorbidities and rotavirus epidemiology differ substantially

from higher income settings. We therefore aimed to investigate risk factors for transmission of rotavirus to household contacts in a semi-urban environment in Blantyre, Malawi.

7.2 Methods

7.2.1 Objectives

1. To identify independent risk factors for transmission of rotavirus infection to household contacts of a symptomatic index child
2. To identify independent risk factors for transmission of rotavirus disease to household contacts of a symptomatic index child

7.2.2 Study design

Data for this study were derived from the prospective cohort study of household contacts of children with symptomatic rotavirus disease recruited as part of the RotaRITE: Transmission Epidemiology Study (RRTE), methods for which are described in detail in chapter 5 (section 5.2, page 154). Briefly, vaccine age-eligible children presenting with acute gastroenteritis to participating study sites were screened, and recruited into the study if eligibility criteria were met. Household contacts were then recruited and followed up for laboratory evidence of subsequent rotavirus infection and for symptoms of gastroenteritis (rotavirus disease).

7.2.3 Study site

Participants for this study were recruited from QECH, Zingwangwa Health Centre, Gateway Health Centre and Madziabango Health Centre.

7.2.4 Study population

Vaccine age-eligible children with acute gastro-enteritis were screened for eligibility criteria, and if eligible were consented to participate in the study. All household contacts of enrolled children who fulfilled eligibility criteria were invited to take part in the study.

7.2.5 Study Procedures

7.2.5.1 Enrolment

Enrolment into the primary study has been described previously (chapter 5, section 5.2, page 154).

7.2.5.2 Data collection and follow up

Index children had detailed data collected at the point of enrolment. Household contacts had baseline data collected at an initial visit, and were asked to report any gastroenteritis symptoms in the preceding 10 days. They then had data on symptoms and a stool sample collected at two time points following onset of symptoms in the index child. The first two samples collected from household contacts members enrolled into the nested intensive cohort (Chapter 6) were also included in this analysis.

7.2.5.3 Sample collection

Each household contact had two samples collected

- Sample 1 at days 5 to 7 after the date of reported onset of symptoms in the index child
- Sample 2 at days 10 to 12 after the date of reported onset of symptoms in the index child

7.2.6 Sample size

The sample size calculation for this study is described in Chapter 5 (section 5.2, page 154).

7.2.7 Laboratory procedures

All stool samples were tested for rotavirus using VP6 qRT-PCR as described in chapters 2 (section 2.3, page 87)and 5 (section 5.2, page 154). Positive results with Ct values >35 were confirmed using an additional assay targeting NSP3.

7.2.8 Statistical methods

Variables were graphed and tabulated to examine distributions of data and look for missing data and inconsistencies. Missing data were excluded from the analysis.

The model for transmission was built using a hierarchal conceptual framework (Fig 7.2)(361). Variables were divided into groups, factors relating to the infectiousness of the symptomatic index child, and susceptibility factors relating to the susceptibility of household contacts to developing rotavirus infection or disease as described above. Susceptibility factors were further divided into proximal susceptibility factors (factors operating at an individual level), and distal susceptibility factors (factors operating at a household level). Individual models were built for each of these 3 groups of co-variates to select risk factors of importance, whilst adjusting for potential confounding. A final model

was then built incorporating all three groups, starting with distal susceptibility factors and then adding in proximal susceptibility factors and finally infectiousness factors. Separate models were built for infection and disease. Models were build using logistic regression models with a random effect to account for clustering at the household level. Akaike's Information Criterion (AIC) are reported for the constituent and final models for infectiousness and disease, respectively.

Model selection was conducted as follows. First, univariable analysis was conducted between the outcome variable and all potential exposure variables in turn. All variables with $p<0.1$ on univariable analysis were then tested for inclusion in the final model. The variable with the largest association (i.e. smallest p value) with the outcome on univariable analysis was the first variable selected for inclusion, and then regression analysis was conducted using this model and each subsequent exposure variable. Nested models were compared using likelihood ratio tests. The variable with the smallest p value from the regression analysis became the newly selected variable and included in the model. This cycle was continued until no more exposure variables were able to be included (because $p>0.1$ when tested). If a variable improved the model it was included, and retained in the model even if it subsequently lost significance.

Co-linearity between co-variates was evaluated based on a-priori knowledge and using pair-wise correlation coefficients where relevant. For pairs of co-linear co-variates, only one of the pair was included in the final model.

This analysis presents the effect of potential risk factors on the likelihood of transmission using odds ratios (OR). If an outcome is rare the OR approximates to the relative risk (RR) (rare disease assumption), however as an outcome becomes more common the OR becomes larger in comparison to the RR(326). As a result the effect size of the odds ratio can be prone to misinterpretation. A sensitivity analysis was therefore performed to estimate the RR of infection and disease in household contacts by fitting a Poisson model using generalised estimating equations (GEE) with exchangeable correlation matrix to account for the household level clustering(362).

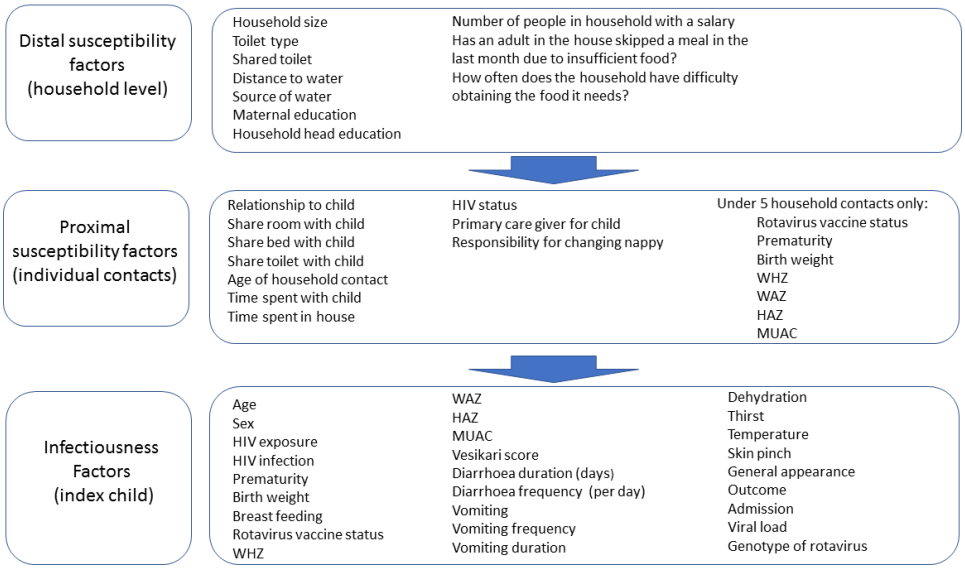


Figure 7.2. Hierarchical framework for constructing model of risk factors for transmission

7.2.9 Definitions

Definitions of infection, disease, index child, household and contact can be found in Chapter 5 (section 5.2, page 154).

7.3 Results

705 household contacts were recruited from 196 index children. The characteristics of these contacts are outlined in chapter 5 (section 5.3.4, page 174). All 705 household members contributed symptom information, and 665 individuals from 188 households had qRT-PCR data. Overall 435/665 (65.4%) of household contacts of infected index children were themselves infected with rotavirus, and 173/188 (92%) households had at least one episode of transmission. 37/698 (5%) household contacts reported rotavirus associated gastroenteritis, which was at least one episode in 33/196 (17%) households.

7.3.1 Risk factors for rotavirus infection

7.3.1.1. Distal susceptibility factors for infection

On univariable analysis, households in which at least one member earned a regular salary had lower odds of transmission than households where no-one earned a r-egular salary, OR 0.47 (95% CI 0.26, 0.86), and households where the household head had higher education showed weak evidence of lower odds of transmission (OR 0.37, 95% CI 0.13, 1.03). Households that obtained water from a tap or a borehole had decreased odds of

transmission compared to households using a well (Table 7.1). Difficulty obtaining sufficient foods for the household to eat was associated with reduced odds of transmission, OR 0.52 (95% CI 0.28, 0.96). On multivariable analysis presence of a household member who earned a salary and difficulty obtaining sufficient foods for the household remained in the model (OR 0.45 [95% CI 0.25, 0.82] and OR 0.49 [95% CI 0.27, 0.90] respectively).

Table 7.1 Distal susceptibility risk factors for transmission of rotavirus infection

	Univariate analysis				Multivariable analysis			
Variable	OR	95% CI	P	N	OR	95% CI	P	N
Household size				665				663
<5	REF							
>5	0.672	0.37, 1.21	0.186					
Toilet type				665				
None	REF							
Simple pit/VIP	0.18	0.02, 1.81	0.146					
Water toilet	0.15	0.01, 2.05	0.154					
Shared toilet				665				
No	REF							
Yes	0.54	0.28, 1.06	0.072					
Distance to water				665				
0-5 mins	REF							
5-30mins	0.91	0.41, 2.01	0.808					
>30 mins	1.07	0.47, 2.44	0.881					
Water source				663				
Well	REF							
Borehole	0.29	0.07, 1.13	0.075					
Shared tap	0.22	0.06, 0.76	0.017					
Tap to house	0.28	0.07, 1.16	0.079					
Maternal education				665				
Primary or less	REF							
Secondary	0.03	0.51, 1.69	0.810					
Higher	0.79	0.19, 3.33	0.744					
Household head education				645				
Primary or less	REF							
Secondary	1.07	0.56, 2.03	0.839					
Higher	0.37	0.13, 1.03	0.058					
Number of adults with salary in household				663				
None	REF				REF			
≥1	0.47	0.26, 0.86	0.014		0.45	0.25, 0.82	0.009	663
Has an adult skipped a meal in the past 2 weeks?				665				
No	REF							
Yes	0.65	0.34,1.27	0.213					
Problems getting food in the past month				665				
No	REF				REF			
Sometimes/often	0.52	0.28, 0.96	0.037		0.49	0.27, 0.90	0.022	663

7.3.1.2 Proximal susceptibility factors for infection

On univariable analysis, other adult relatives or child household contacts were less likely than mothers to become infected with rotavirus (OR 0.28 [95% CI 0.16, 0.49]) and 0.49 [95% 0.28, 0.81], respectively). Sleeping in the same room as the index child was weakly associated with an increased risk of transmission (OR 1.46 [95% CI 0.85, 1.93]). Spending increasing amounts of time in the house and with the index child were both associated

with an increased risk of transmission(Table 7.2), as was being the primary care giver (OR 2.25, 95% CI 1.43, 3.55) and frequently changing the child's nappy (OR 3.32, 95% CI 1.92. 5.75). Rotavirus vaccination status was not associated with risk of infection (OR 1.45 [95% CI 0.12, 17.46] and 1.51 [95% CI 0.60, 3.80] for one or two doses respectively. Once adjusted for relationship to the child on multivariable analysis no other risk factor remained significantly associated with rotavirus of infection.

Table 7.2. Proximal susceptibility risk factors for transmission

	Univariate analysis				Multivariable analysis			
	OR	95% CI	P	N	OR	95% CI	P	N
Relationship to child								
Mother	REF			665	REF			665
Other adult relative	0.28	0.16, 0.49	0.000		0.28	0.16, 0.49	0.000	
Child contact	0.49	0.28, 0.81	0.005		0.49	0.28, 0.81	0.005	
Sleep in same room as index child				665				
No	REF							
Yes	1.46	0.95,2.25	0.083					
Share bed with index child				665				
No	REF							
Yes	1.28	0.85, 1.93	0.240					
Share toilet with index child				665				
Never	REF							
Sometimes/often	1.92	0.57,6.57	0.294					
Household member age				664				
<5 years	REF							
5-15 years	0.85	0.44,1.65	0.639					
15-45 years	0.87	0.47,1.59	0.644					
45+ years	0.57	0.14,2.31	0.435					
Time spent with index child				665				
All day	REF							
Half day	0.70	0.44, 1.13	0.144					
Evening only/no time	0.52	0.31, 0.87	0.012					
Time spent in house				665				
All day	REF							
Half day	0.70	0.44,1.12	0.139					
Evening only/no time	0.48	0.29, 0.80	0.005					
HIV status								
Uninfected	REF							
Infected	1.37	0.40,4.67	0.619	325				
Primary care giver for child				665				
No								
Yes	2.25	1.43, 3.55	0.000					

Responsible for changing nappy				665
Never/sometimes	REF			
Always/often	3.32	1.92,5.75	0.000	
Child/NA	1.72	1.06,2.79	0.029	
Children under years 5 only				
RV1 doses				81
0	REF			
1	1.45	0.12,17.46	0.768	
2	1.51	0.60, 3.80	0.381	
Premature				
No	REF			
Yes	0.43	0.14, 1.30	0.135	
Birth weight (Kgs)	1.67	0.62, 4.56	0.313	80
WAZ	1.16	0.85, 1.58	0.359	82
WHZ	1.02	0.79, 1.34	0.827	73
HAZ	1.17	0.85, 1.62	0.346	73
MUAC	0.90	0.73, 1.11	0.327	87

7.3.1.3 Infectiousness risk factors for infection

Several clinical features of rotavirus disease in the index child acted were identified as univariable risk factors for transmission of rotavirus infection within the home (Table 7.3). The presence of vomiting was significantly associated with transmission (OR 3.40, 95% CI 1.20,9.64), as was requirement for admission (OR 2.29, 95% CI 1.29, 4.06) and increasing Vesikari score (OR 1.17, 95% CI 1.05, 1.30). Several other markers of disease severity in the index child showed a trend towards increased risk of transmission, but were not significant at the 5% level. These included increasing duration of vomiting and frequency of vomiting and increasing duration and daily frequency of diarrhoea, the presence of dehydration, and death before discharge from hospital. Other factors which showed weak evidence of an association with increased risk of transmission were increasing height for age Z score (OR 1.12, 95% CI 1.00, 1.27) and increasing MUAC (OR 1.25, 95% CI 1.00, 1.55). On multivariable analysis age in months, sex and viral load were included in the multivariable model a-priori. Increasing Vesikari score and increasing MUAC were also identified as independent risk factors for transmission (OR 1.16 [95% CI 1.04, 1.29] and OR 1.32 [95% CI 1.05, 1.68], respectively).

Table 7.3. Infectiousness risk factors for rotavirus infection

Variable	Univariate analysis OR	95 % CI	P value	N	Multivariable analysis OR	95% CI	P value	N
Vesikari score	1.17	1.05, 1.30	0.004	652	1.16	1.04,1.29	0.007	625
Diarrhoea duration (days)				665				
1-3	REF							
5	1.15	0.34,3.85	0.824					
≥6	2.23	0.63,7.94	0.215					
Diarrhoea episodes*				665				
1-4	REF							
5	1.74	0.70,4.32	0.232					
≥6	2.28	0.92, 5.69	0.077					
Vomiting				665				
No	REF							
Yes	3.40	1.20, 9.64	0.021					
Vomiting duration (days)				612				
1	REF							
2	0.84	0.32, 2.15	0.710					
≥3	1.65	0.68, 4.00	0.272					
Vomiting frequency*				612				
<5	REF							
≥5	1.68	0.88, 3.21	0.119					
Temperature (rectal, °C)				655				
37.1-38.4	REF							
38.5-38.9	0.54	0.26, 1.11	0.105					
≥39.0	0.68	0.34, 1.37	0.282					
Skin pinch				665				
Normal	REF							
Goes back slowly	1.94	1.01,3.73	0.046					
Goes back very slowly	1.83	0.76,4.39	0.179					
General appearance				665				
Alert	REF							
Restless	0.97	0.53,1.78	0.921					
Unconscious	0.91	0.34,2.46	0.850					
Thirst				665				
No	REF							
Thirsty	1.87	0.85, 4.11	0.119					
Drinks poorly	2.02	0.66, 6.15	0.215					
Dehydration				665				
None	REF							
Some	1.47	0.62,3.51	0.385					
Severe	1.35	0.50, 3.67	0.560					
Admission				665				
No	REF							
Yes	2.29	1.29,4.06	0.005					
Outcome				665				
Home	REF							
Died	2.45	0.10, 60.06	0.583					
Sex								
	REF							
Male	1.00	0.56,1.77	0.988	665	1.00	0.56,1.79	0.980	625
Age in months	1.00	0.96, 1.04	0.941	665	1.00	0.95,1.04	0.872	625

Log viral copy numbers	1.05	0.95, 1.15	0.346	643	1.04	0.95,1.15	0.980	625
HIV exposed				665				
No	REF							
Yes	1.44	0.58,3.57	0.430					
HIV infected*	-	-	-					
Premature				665				
No	REF							
Yes	0.71	0.16,3.19	0.660					
Birth weight (Kgs)	1.47	0.95, 2.29	0.086	625				
Ever breastfed								
No	REF							
Yes	9.60	0.24,378.8	0.228	665				
Completed rotavirus vaccination				665				
No	REF							
Yes	1.71	0.10, 29.64	0.711					
Adjusted WHZ	0.89	0.75,1.06	0.206	662				
Adjusted WAZ	1.21	0.95, 1.54	0.122	662				
Adjusted HAZ	1.12	1.00, 1.27	0.053	648				
MUAC	1.25	1.00, 1.57	0.055	660	1.32	1.05,1.68	0.019	625
Genotype				663				
G1P8	REF							
G2P4	0.44	0.20,0.96	0.038					
G2P6	0.50	0.19,1.28	0.148					
G12P6	1.24	0.34, 4.56	0.747					
Other type	0.54	0.24,1.25	0.153					

*maximum number per day **model unable to converge

7.3.1.4 Final model for risk factors for rotavirus infection

The fully adjusted model had a lower Akaike's Information Criterion (AIC) than the constituent models (Table 7.4). In the fully adjusted model, the presence of at least one adult in the household with a salary continued to be associated with reduced odds of rotavirus transmission (OR 0.41, 95% CI 0.22, 0.75)(Table 7.5) Other adult household members and children remained significantly less likely to be infected than mothers, and increasing Vesikari score in the index child was associated with increasing odds of transmission to contacts (OR 1.16, 95% CI 1.04, 1.29). The relative risk of transmission is also reported, with an increased risk of transmission of 1.04 (95% CI 1.01, 1.07) per unit increase in Vesikari score and 1.08 (95% CI 1.02, 1.14) per unit increase in MUAC. A decreased risk of 0.82 (95% CI 0.72, 0.93) is described for households in which at least one member had a regular salary, and of 0.75 (95% CI 0.65, 0.86) and 0.83 (95% CI 0.74, 0.92) compared to mothers for other adults and children in the household.

Table 7.4 Akaike's Information Criterion for multivariate models for risk factors for infection

	Akaike's Information Criteria
Distal susceptibility risk factors for infection	791
Proximal susceptibility risk factors for infection	784
Infectiousness risk factors for infection	748
Final model for infection	721

Table 7.5 Final model for risk factors for rotavirus infection

Variable	OR	P value	95% CI	RR	P Value	95% CI	N
							623
Vesikari	1.16	0.009	1.04, 1.29	1.04	0.008	1.01, 1.07	
Age in months	1.00	0.851	0.95, 1.04	1.00	0.814	1.00, 1.01	
Sex	REF						
Male	1.00	0.965	0.55, 1.76	0.98	0.788	0.85, 1.13	
Log viral copy number	1.05	0.282	0.96, 1.16	1.01	0.380	0.99, 1.04	
Number of adults with salary in household							
None	REF						
≥1	0.41	0.004	0.22, 0.75	0.82	0.002	0.72, 0.93	
Problems getting food in the past month (%)							
No	REF						
Sometimes/often	0.69	0.240	0.37, 1.28	0.90	0.213	0.76, 1.06	
Relationship with child							
Mother	REF						
Other adult relative	0.28	0.000	0.16, 0.50	0.75	0.000	0.65, 0.86	
Child contact	0.40	0.001	0.24, 0.68	0.83	0.001	0.74, 0.92	
MUAC	1.37	0.009	1.08, 1.76	1.08	0.004	1.02, 1.14	

7.3.2 Risk Factors for disease

7.3.2.1 Distal susceptibility factors for disease

The only distal susceptibility factor associated with the risk of rotavirus disease was toilet type, where using a pit toilet or a water toilet were associated with a reduced odds of rotavirus disease compared to having no toilet (OR 0.16 {95% CI 0.04, 0.68] and OR 0.10 [95% CI 0.01, 1.26], respectively) (Table 7.6).

Table 7.6 Distal susceptibility factors for disease

	Univariate analysis				Multivariable analysis			
Variable	OR	95% CI	P	N	OR	95% CI	P	N
Household size				698				
<5	REF							
>5	0.75	0.36, 1.54	0.429					
Toilet type				698				698
None	REF							
Simple pit/VIP	0.16	0.04, 0.68	0.014		0.16	0.04, 0.68	0.014	
Water toilet	0.10	0.01, 1.26	0.075		0.10	0.01, 1.26	0.075	
Shared toilet				698				
No	REF							
Yes	0.68	0.33, 1.43	0.306					
Distance to water				698				
0-5 mins	REF							
5-30mins	2.30	0.74, 7.14	0.150					
>30 mins	1.41	0.43, 4.66	0.557					
Water source				696				
Well	REF							
Borehole	5.51	0.68, 44.34	0.109					
Shared tap	2.24	0.29, 17.42	0.440					
Tap to house	2.54	0.28, 22.82	0.406					
Maternal education				698				
Primary or less	REF							
Secondary	0.92	0.44, 1.94	0.832					
Higher	0.72	0.09, 6.12	0.765					
Household head education	0.97	0.70, 1.33	0.830	678				
Primary or less	REF							
Secondary	1.11	0.49, 2.54	0.800					
Higher	0.62	0.12, 3.17	0.569					
Number of adults with salary in household				696				
None	REF							
≥1	0.65	0.32, 1.31	0.227					
Has an adult skipped a meal in the past 2 weeks?				698				
No	REF							
Yes	0.91	0.38, 2.15	0.822					
Problems getting food in the past month (%)				698				
No	REF							
Sometimes/often	1.08	0.50, 2.29	0.851					

7.3.2.2 Proximal susceptibility factors for disease

Age of household contact was significantly associated with odds of disease. Odds of disease were highest in those aged under 5 years, and all other age groups were at significantly reduced odds of disease in comparison (OR 0.12 [95% CI 0.04, 0.42] for children aged 5-15 years, 0.34 [95% CI 0.15, 0.75] for contacts aged 15-45 years and 0.36 [95% CI 0.04, 3.21] for contacts aged 45 years or older (Table 7.7). The model investigating

the association between vaccination status of children in the households and risk of disease did not achieve convergence. On multivariable analysis only age of the household contact remained significant.

Table 7.7. Proximal susceptibility factors for disease

	Univariate analysis				Multivariable analysis			
	OR	95% CI	P	N	OR	95% CI	P	N
Relationship to child				698				
Mother	REF							
Other adult relative	0.90	0.37, 2.19	0.810					
Child contact	0.91	0.41, 2.03	0.815					
Share bedroom with index child				698				
No	REF							
Yes	1.89	0.88, 4.02	0.104					
Share bed with index child				698				
No	REF							
Yes	1.65	0.82, 3.31	0.163					
Shared toilet with child				698				
No	REF							
Sometimes/always	4.08	1.00, 17.01	0.053					
Household member age				696				696
<5 years	REF				REF			
5-15 years	0.12	0.04, 0.42	0.001		0.12	0.04, 0.42	0.001	
15-45 years	0.34	0.15, 0.75	0.008		0.34	0.15, 0.75	0.008	
45+ years	0.36	0.04, 3.21	0.359		0.36	0.04, 3.21	0.359	
Time spent with index child				698				
All day	REF							
Half day	0.60	0.26, 1.41	0.247					
Evening only/no time	1.18	0.53, 2.64	0.689					
Time spent in house				698				
All day	REF							
Half day	0.67	0.30, 1.53	0.343					
Evening only/no time	1.07	0.47, 2.45	0.870					
HIV status								
Uninfected	REF			335				
Infected	0.91	0.17, 4.99	0.916					
Primary care giver for child								
No	REF			698				
Yes	1.13	0.55, 2.33	0.737					
Responsible for changing nappy				698				
Never/sometimes	REF							
Always/often	1.02	0.42, 2.49	0.969					
Child/NA	0.97	0.42, 2.21	0.937					
Household contacts under 5 years only								
Rotavirus vaccine doses*								
0	REF							
1	-							
2	-							
Premature								
No	REF			105				
Yes	1.13	0.22. 5.71	0.884					

Birth weight(Kgs)	1.37	0.46, 4.09	0.572	82
WAZ	0.94	0.63, 1.40	0.745	84
WHZ	0.98	0.70, 1.36	0.897	75
HAZ	1.38	0.88, 2.17	0.157	75
MUAC	0.23	0.06, 1.54	0.900	88

* model unable to converge

7.3.2 3 Infectiousness risk factors for rotavirus disease

Disease severity in the index child was associated with increasing odds of disease in household contacts. There was a positive association between Vesikari score and odds of disease (1.27 [95% CI 1.08, 1.48]). Vomiting frequency was associated with increased odds of disease (OR 2.59, 95% CI 1.25, 5.37), as was admission to hospital (OR 3.27, 95% CI 1.37, 7.80). Weak evidence of an association with odds of disease was described for vomiting duration of 3 days or more (OR 6.22, 95% CI 0.81, 47.66), severe dehydration (OR 7.53, 95% CI 0.93, 61.19)and rotavirus strain in the index child. In terms of rotavirus genotype, compared to G1P[8] strains G2P[4], G2P[6] and G12P[6] strains in the index child were associated with reduced odds of disease transmission (OR 0.38 [95% CI 0.14, 1.04], 0.50 [95% CI 0.14, 1.84] and 1.26 [0.38, 4.21]), respectively). Age in months and viral load were included in the multivariable model a-priori. In addition to these Vesikari score was identified as an independent risk factor for disease in household contacts (Table 7.8).

Table 7.8 Infectiousness factors for disease

	Univariate analysis				Multivariable analysis			
Variable	OR	95 % CI	p	N	OR	95% CI	P	N
Vesikari	1.27	1.08, 1.48	0.004	685	1.29	1.08, 1.55	0.005	663
Diarrhoea duration				698				
1-3	REF							
5	0.41	0.05, 3.30	0.405					
≥6	1.73	0.52, 5.69	0.369					
Diarrhoea episodes				698				
1-4	REF							
5	1.07	0.28, 4.12	0.926					
≥6	1.59	0.43, 5.87	0.485					
Vomiting				698				
No	REF							
Yes	3.43	0.43, 27.28	0.244					
Vomiting duration				642				
1	REF							
2	4.56	0.56, 37.13	0.156					
≥3	6.22	0.81, 47.66	0.078					
Vomiting frequency*				642				
1-4	REF							
≥5	2.59	1.25, 5.37	0.010					
Temperature				689				
37.1-38.4	REF							
38.5-38.9	1.07	0.47, 2.39	0.888					
≥39.0	0.60	0.24, 1.48	0.265					
Skin pinch				698				
Normal	REF				REF			
Goes back slowly	1.07	0.44, 2.64	0.880		0.44	0.16, 1.22	0.115	
Goes back very slowly	2.70	0.96, 7.56	0.059		0.95	0.31, 2.93	0.926	
Sunken eyed				698				
No	REF							
Yes	3.62	0.47, 27.80	0.217					
General appearance				698				
Alert	REF							
Restless	1.44	0.68, 3.08	0.342					
Unconscious	1.13	0.29, 4.37	0.856					
Thirst				698				
No	REF							
Thirsty	6.19	0.82, 46.80	0.077					
Drinks poorly	6.19	0.65, 58.69	0.112					
Dehydration				698				
None	REF							
Some	4.27	0.55, 32.88	0.163					
Severe	7.53	0.93, 61.19	0.059					
Admission				704				
No	REF							
Yes	3.27	1.37, 7.80	0.008					
Outcome				698				
Home	REF							
Died	4.78	0.40, 56.49	0.215					
Sex				698				

Female	REF						
Male	1.69	0.81, 3.56	0.165				
Age in months	1.00	0.95, 1.06	1.00	698	1.00	0.95, 1.05	0.916
Log copy viral numbers	0.98	0.87, 1.11	0.727	676	0.98	0.86, 1.11	0.718
HIV exposed				698			
No	REF						
Yes	1.85	0.73, 4.69	0.193				
HIV infected				188			
No	REF						
Yes	2.93	0.19, 46.14	0.446				
Premature				704			
No	REF						
Yes	2.53	0.61, 10.49	0.202				
Birth weight (kgs)	0.76	0.44, 1.29	0.307	651			
Ever breastfed**							
No	REF						
Yes	-	-	-				
Completed rotavirus vaccine**							
No	REF						
Yes	-	-	-				
Adjusted WHZ	0.91	0.73, 1.13	0.389	695			
Adjusted WAZ	0.90	0.67, 1.20	0.461	695			
Adjusted HAZ	0.98	0.85, 1.14	0.839	680			
MUAC	0.97	0.74, 1.28	0.857	693			
Genotype				696			
G1P8	REF						
G2P4	0.38	0.14, 1.04	0.060				
G2P6	0.50	0.14, 1.84	0.297				
G12P6	1.26	0.38, 4.21	0.706				
Other type	1.17	0.50, 2.75	0.710				

*maximum number per day **model unable to converge

7.3.2.4 Final model for risk factors for disease.

Akaike's Information Criterion (AIC) for the multivariate models for risk factors for infection can be seen in Table 7.9, and as with the model for infection, the fully adjusted model had the lowest AIC. Once fully adjusted, Vesikari score in the index child remained positively associated with increased odds of disease in household contacts (OR 1.31, 95% CI 1.08, 1.58). Age of household contact was also found to be an independent risk factor for disease (Table 7.10). The relative risk for disease is also estimated. In this case the OR and RR for disease are similar, as the overall prevalence of rotavirus disease was relatively uncommon (5%) (Table 7.10).

Table 7.9 Akaike's Information Criterion for multivariate models for risk factors for disease

	Akaike's Information Criteria
Distal susceptibility risk factors for disease	290
Proximal susceptibility risk factors for disease	284
Infectiousness risk factors for disease	280
Final model for disease	273

Table 7.10 Final model for risk factors for rotavirus disease

Disease	OR	P value	95% CI	RR	P value	95% CI	N
							661
Age in months	1.02	0.437	0.97, 1.08	1.02	0.396	0.98, 1.06	
Log viral copy numbers	1.00	0.990	0.88, 1.13	1.00	0.897	0.92, 1.10	
Toilet type							
None	REF						
Simple pit/VIP	0.47	0.370	0.09, 2.47	0.62	0.310	0.24, 1.57	
Water toilet	0.27	0.336	0.02, 3.94	0.34	0.341	0.04, 3.12	
Skin pinch							
Normal	REF						
Goes back slowly	0.47	0.159	0.16, 1.35	0.51	0.265	0.16, 1.66	
Goes back very slowly	0.95	0.928	0.29, 3.05	0.93	0.907	0.26, 3.14	
Household member age							
<5 years	REF						
5-15 years	0.12	0.001	0.03, 0.44	0.15	0.001	0.05, 0.48	
15-45 years	0.34	0.015	0.14, 0.81	0.38	0.005	0.19, 0.75	
45+ years	0.37	0.376	0.04, 3.36	0.42	0.400	0.06, 3.13	
Vesikari score	1.31	0.005	1.08, 1.58	1.27	0.001	1.09, 1.48	

7.4 Discussion

This study found that increasing disease severity in the index child is associated with increased odds of both rotavirus infection and clinical disease in household contacts. Although the association between SAR and disease severity is modest, this finding could be important, as it suggests that reducing symptom severity in an index child may have potential to reduce rotavirus transmission. However, interestingly and contrary to what was hypothesised, the relationship between disease severity and transmission does not appear to be mediated by viral load.

Increasing Vesikari score is associated with increased odds of rotavirus transmission. Vesikari score is an ordinal score of disease severity which is typically used to define severe disease for the purposes of vaccine studies and is a composite end point made up of several different markers of disease severity (Chapter 5, section 5.2, page 154). Although several component variables of disease severity such as vomiting and admission to hospital were associated with odds of transmission at a univariate level, none were independently associated with transmission once Vesikari score was included in the model, suggesting that Vesikari score is a good marker of disease severity in our setting. Interestingly hospital admission was strongly predictive of the risk of transmission for both infection and disease. This is somewhat counter-intuitive as once admitted children were removed from households and contact with susceptible household members. This suggests that most transmission within households is occurring soon after the index case develops illness, before presentation to healthcare. This is supported by the significant decline in rotavirus viral load in household contacts over time since symptom onset in index children observed in chapter 6 (section 6.3.3, page 201). It also suggests that admission is an objective measure of disease severity, particularly in a context such as Malawi where literacy rates are low and providing accurate estimates of frequency and duration of symptoms can be challenging.

Despite the findings described in chapter 6 (section 6.3.1.1, page 196) that disease severity is positively associated with viral load in the index child and the association between disease severity and odds of transmission described in this chapter, there was no association between viral load and risk of transmission. This is contrary to findings in Ecuador, where children with a Ct value of <15 on qRT-PCR were more likely to transmit than those with lower viral loads(106). In our setting the mechanism of increased odds of transmission with increasing disease severity may reflect increased environmental contamination or increased opportunity for contact with infectious material in the household, rather than an increased density of viral shedding per-se. Possibly once children are symptomatic in this cohort, viral loads are too homogenous to identify any difference based on viral load, or differences in living conditions and sanitation result in symptoms being the more important driver of transmission.

In terms of other infectiousness risk factors for transmission, the positive association with increasing MUAC in the index child is intriguing. The association between MUAC and transmission is corroborated by the finding of reduced odds of transmission in households where food is scarce, although this loses significance in the fully adjusted model. A study

in Bangladesh by Verkerke et al found that malnutrition was protective against rotavirus disease in infants, so it is possible that MUAC in the index child is a proxy for the overall nutritional status of the household, and that poor nutrition in the household is protective against rotavirus infection(363). These findings seem contrary however to published associations between increased severity of diarrhoeal disease and SAM(345), and the negative association between WHZ and viral shedding density observed in chapter 6 (section 6.3.1.1, page 196). It is possible that there are separate mechanisms at play, and that as postulated by Verkerke at al(363), malnutrition is protective against infection with rotavirus because of an associated enteropathy, but if infection does occur children develop more severe disease due to global immune impairment(364).

Household level susceptibility factors associated with transmission of infection include the lack of a regular salary in the household. This is likely to be a proxy for relative poverty, and there are numerous reasons why relative poverty could result in increased transmission, including crowding, sanitation, carer education levels, and many other unmeasured factors. This finding is supported in the univariate analysis by the reduced risk of transmission in households where the household head has higher level education, although this association loses significance when adjusted for salary in the multivariable model. The absence of a toilet was significantly associated with the risk of disease in household contacts in the multivariable disease susceptibility model, but is no longer significant in the fully adjusted final model for disease. This is likely to reflect low levels of hygiene and sanitation which could increase the risk of transmission, or form another proxy for poverty.

In terms of proximal risk factors for transmission of infection, the main risk factor appears to be contact with the index child, with sharing a bedroom with the child, spending time with the child or in the house, and changing the nappy all significantly increasing the risk of transmission on univariate analysis. Vaccine status of household contacts was not associated with risk of infection. This could be a result of lack of power, as vaccine status was only asked of children under 5 years of age resulting in large numbers of missing data for this variable. However it is also plausible that vaccination does not protect against infection with rotavirus. The relatively small number of contacts with data on vaccine status, combined with the small number of disease episodes is the most likely explanation for the lack of convergence of the model investigating vaccine status and disease. The single independent proximal susceptibility risk factor for infection is relationship with the child, which incorporates all of the above plus likely several other unmeasured factors.

Age of the household contact was not associated with risk of infection, which is in direct contrast with the proximal risk factors for disease where age of the household contact is strongly associated with odds of disease. This suggests that in Malawi individuals develop immune protection against clinical disease with increasing age and repeated exposure, but not against infection. This differs from age distributions of asymptomatic rotavirus observed in the UK in a case control study of diarrhoea aetiology, where the prevalence of asymptomatic infection declined with increasing age(19), but reflects the patterns seen in the study of household transmission in Ecuador(106), and in the asymptomatic community control households in this study (Chapter 5, section 5.3.6.2, page 178). This could be explained by different exposure patterns; household contacts in this study and in Ecuador all have a known exposure, and adults in Malawi are more likely to have regular contact with children than adults in the UK in general, but it is possible it could also reflect differences in immunity between populations. Children under five were at the highest risk of rotavirus disease despite the fact that over 50% of this age group had received two doses of rotavirus vaccine (41/79, Table 5.7, page 174) and the older age groups were not yet vaccine age eligible. It is possible that the association between age and risk of rotavirus disease was even stronger prior to rotavirus vaccine introduction.

7.4.1 Limitations

Due to very high vaccine coverage it was not possible to investigate whether vaccine status in the index child had any effect on risk of transmission to household contacts. Although the sample size for this study is large, there were few episodes of rotavirus disease. The regression analysis for disease will therefore be underpowered, and should be interpreted with some caution in light of this(365). The factors contributing to transmission are complex and though this study accounts for this as best as possible with a structured approach to the analysis, it is impossible to collect data on all possible confounding variables and there is a risk of unmeasured confounding which should be considered when interpreting the study findings. Finally the population which accesses government health care in Malawi, while representative of the majority of the urban population in Malawi, is extremely poor, and possibly too homogenous to identify risk factors in transmission which relate to poverty or water or sanitation.

7.4.2 Conclusions, implications and future study

The observation that disease severity predicts risk of transmission for both infection and disease in this setting is potentially important as it suggests that vaccine mediated

reduction in disease severity has potential to reduce rotavirus transmission to household contacts of index cases. It is unfortunately not possible to investigate the effect of vaccine exposure on transmission in this study because of the high vaccine coverage in Blantyre, however future studies could model the potential effect of vaccine on transmission using these data to inform model parameterisation.

As in chapter 5, this analysis highlights the lack of immunity to infection with increasing age in household contacts. It is not clear if this reflects sub-optimal immune response to repeated exposure, or if it relates to contact patterns or intensity of exposure. Further studies could explore this by investigating contact patterns, and incorporating high income populations with similar exposure patterns.

The negative association between MUAC and odds of transmission is not fully understood, and although it could simply reflect sampling error, the fact that it is corroborated by the association with food scarcity in the household and by observations from Bangladesh suggests that further studies are needed to explore the relationship between nutritional status and rotavirus disease.

This study highlights the complex factors contributing to rotavirus transmission in low income settings and identifies key risk factors for transmission, including disease severity in the index child. It will form the basis for more complex analysis investigating the potential effect vaccine could play on rotavirus transmission in our setting.

8. Horizontal transmission of rotavirus vaccine virus to household contacts

8.1 Introduction

Chapter four outlined the potential importance of indirect or transmission-mediated vaccine effects in the overall population level impact of rotavirus vaccine, in the context of high rotavirus disease burden and reduced vaccine effectiveness in low-income countries. Indirect effects have been documented from high income countries, but the presence and extent of rotavirus vaccine indirect effects in low-income countries in Africa and Asia remain unknown. This chapter focusses on the role that horizontal transmission of vaccine virus may play in rotavirus vaccine indirect effects in low-income settings.

The mechanisms behind observed indirect effects of rotavirus vaccine are not fully understood but may include reduction in transmission of wild-type rotavirus as discussed in previous chapters, and/or horizontal transmission of vaccine-virus following excretion in stool of vaccinated infants(215,360). Horizontal transmission of vaccine virus to unvaccinated individuals has been well described with another enterally-administered vaccine, oral polio vaccine(366), but the role of such transmission in the generation of rotavirus vaccine indirect effects is not yet established.

Both current globally licensed rotavirus vaccines (RV1 and RV5) are live oral vaccines which mimic natural infection, and replicate in the gastrointestinal tract before being shed in the stool. To date, most data on vaccine virus shedding have come from high income countries, and shedding has most commonly been identified using enzyme-linked immunosorbent assay (EIA) in combination with virus culture in MA104-cells, both of which are less sensitive than molecular methods(341) and likely underestimate the frequency of shedding. A review of studies reporting vaccine shedding post vaccination (all defined using EIA and cell culture) reported rates of shedding that varied between 0 to 80% for RV1, with the level of viral shedding typically greater following dose 1 than after dose 2; these studies also described more frequent vaccine virus shedding following RV1 compared to RV5. In the same review, eight studies investigated transmission of vaccine virus to placebo recipients, and five episodes of transmission were described from two of these studies. One study reported 3/50 (4%) episodes of transmission and the other 2/78 (2.6%)(243). Horizontal transmission of RV5 has also been described in siblings exposed to vaccinated infants in the US(367), and of RV1 in twin siblings from a placebo randomised controlled trial in the Dominican Republic(244). To my knowledge, there are

only two studies which have used PCR to detect vaccine virus shedding. The first study from Taiwan described shedding frequency in vaccinated infants and reported shedding rates of 80-90% following both RV1 and RV5 vaccine, with shedding of greater viral density following RV1 compared to RV5. The second, from Japan, reported horizontal transmission of vaccine virus to contacts of RV1 and RV5 vaccinated infants in a foster home with only one in 23 unvaccinated infants in a foster home shedding detectable vaccine virus(368,369).

Transmission of rotavirus vaccine virus to close contacts has not previously been investigated in low-income settings where differences in disease epidemiology, socio-economic factors and the underlying burden of co-morbidities mean that the risk of transmission of vaccine virus is likely to differ compared to that observed in high and middle income countries. Differences among populations has potential to effect both the frequency and density of viral shedding in the vaccinated infant and therefore impact on their infectiousness to close contacts, and to affect the susceptibility of close contacts to infection.

Frequency and density of vaccine virus shedding is thought to reflect vaccine "take", or strength of vaccine response(131,370,371). There are widespread data to suggest that rotavirus vaccine is less immunogenic in low-income compared to high-income countries(298), and as a result vaccine virus shedding could be less frequent and occur at a lower level than that observed in high income settings. Similarly in countries where oral polio vaccine (OPV) is still used, concomitant administration of OPV may inhibit rotavirus vaccine replication and reduce shedding(372). Contrary to this, prolonged rotavirus shedding has been described in HIV infected children following rotavirus disease, and in children with hereditary immunodeficiencies after rotavirus vaccination(260,373), which could mean that in countries with a high frequency of lowered immunity due to HIV or malnutrition, post vaccine viral shedding may be prolonged. This is supported by the finding described in chapter 6 (section 6.4, page 206)that the median duration of rotavirus shedding following symptomatic disease in Malawi was considerably longer than previously described in Australia(254). Any differences in viral shedding in infants from low-income settings could therefore contribute to an increase or a decrease in infectiousness, depending on the overriding effect.

In terms of susceptibility factors, it seems likely that close contacts of vaccinated infants in low-income countries may be more susceptible to infection with vaccine virus.

Crowding in the household, and poor water and sanitation could increase the risk of exposure to rotavirus vaccine virus for unvaccinated contacts, and increased background prevalence of co-morbidities such as malnutrition and HIV could increase risk of infection once exposed. Risk of transmission is likely to vary based on background immunity to rotavirus, which typically increases with age(38), so we might expect the likelihood of infection with rotavirus vaccine virus to decline with increasing age. This has not previously been explicitly investigated, including in high or middle income settings.

Understanding the extent of horizontal transmission of vaccine virus is essential for understanding any potential contribution to rotavirus vaccine indirect effects. This study therefore aimed to investigate the proportion of nearest-aged siblings and other household members exposed to an infant vaccinated with RV1 who subsequently shed vaccine type virus in Malawi, a very low-income country in sub-Saharan Africa. RV1 was introduced to the national immunization programme in Malawi in October 2012.

8.2 Methods

8.2.1 Objectives

1. To establish the proportion of household members exposed to a RV1 vaccinated infant who develop shedding of vaccine virus.
2. To identify risk factors for horizontal transmission of vaccine virus within a household

8.2.2 Study design

This was a prospective cohort study designed to investigate horizontal transmission of rotavirus vaccine virus to household contacts of infants receiving monovalent rotavirus vaccine in Blantyre, Malawi

8.2.3 Study site

This study was conducted at Zingwangwa Health Centre (HC), Blantyre District, Malawi where approximately 80 infants attend for vaccination each month.

8.2.4 Study population

This study recruited household contacts of infants presenting to Zingwangwa Health Centre, Blantyre, Malawi for routine rotavirus vaccine delivered as part of the Expanded Programmed on Immunization (EPI). In Malawi the monovalent rotavirus vaccine is used (Rotarix™) with two oral doses delivered at 6 and 10 weeks of age.

8.2.5 Study procedures

8.2.5.1 Integration with other studies

Infants enrolled in the study were already recruited into a study of predictors of vaccine response (the RotaRITE response to immunization study (RotaRITE:RI). Infants recruited into the RotaRITE:RI study had an initial baseline stool taken pre-vaccine, and then serial stool samples were collected and tested for vaccine virus using qRT-PCR at 2,4,6,8 and 10 days after vaccination. Demographic and clinical data were also collected for each infant at each visit. Clinical and laboratory data from the RotaRITE:RI study were shared with the Horizontal Transmission study following written parental consent.

8.2.5.2 Recruitment of households

Infants were recruited into the RotaRITE:RI study prior to, or on the same day as, their first rotavirus vaccination. Infants who were consented to participate in the RotaRITE:RI study were then invited to take part in the Horizontal Transmission study. Once consent of the parent or guardian was obtained, the household contacts of the infant were approached and asked for their consent or assent to take part in the Horizontal Transmission study. Consent and assent procedures followed the same guidelines as outlined in detail in chapter 5 (section 5.2, page 154) for the primary study.

8.2.5.3 History taking

Field workers collected data on past medical history including HIV status, current symptoms and contact patterns with the vaccinated infant for each household member. Rotavirus vaccine history was collected from children under the age of 5 years. Symptom questionnaires were conducted at the time of each sample collection (Table 8.1).

8.2.5.4 Sample collection

Once household contacts were consented, household members were given stool containers, and mothers were asked to bring baseline stool samples for the household when they attended the clinic for the first dose of rotavirus vaccine. If the infant was recruited on the day of administration of the first dose of vaccine, where possible baseline stool samples were collected from contacts within 24 hours of vaccine receipt. The baseline stool sample was followed by collection of a single stool sample at 8-10 days following each dose of RV1 in the infant. Samples were collected from households by the study team, or family members brought the samples to the health centre themselves. In total 3 stool samples were collected from each household recruit (Fig 8.1). The purpose

of the baseline stool sample was to act as a pre-exposure control sample for each study participant.

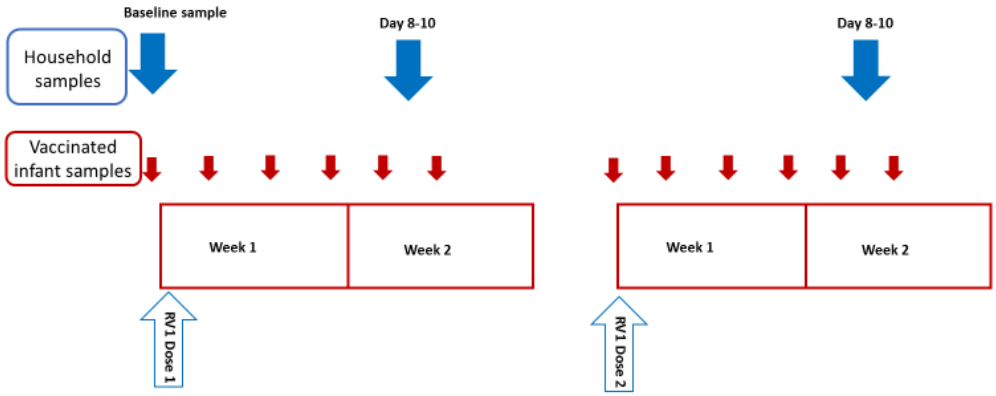


Figure 8.1 Sample collection for the horizontal transmission study

Table 8.1. Overview of processes to be carried out at each visit – households of vaccinated children

	Visit 1	Visit 2	Visit 3
Recruitment of household members	✓		
Adult and child questionnaire	✓		
Symptom Questionnaire		✓	✓
Containers left for stool sample	✓	✓	
Collection of stool sample	✓	✓	✓

8.2.6 Sample size

The sample size required for this study was calculated to estimate the proportion of nearest-aged siblings with detectable vaccine virus shedding following exposure to a vaccinated infant. Sample sizes were calculated to estimate the number of discordant pairs required for a two-sided test McNemar's test of paired proportions. A discordant pair refers to a child who was a non-shedder pre-exposure, who subsequently goes on to shed vaccine virus, or vice-versa. If 95% of discordant pairs were assumed to arise from children who did not shed vaccine type virus pre-exposure, but who subsequently shed

post exposure, 9 discordant pairs were estimated to be required to detect a significant difference between the proportion of household contacts shedding vaccine virus before and after exposure to rotavirus vaccine with 80% power and an alpha of 0.05.

From recent qRT-PCR data in the same population between 60-80% of infants receiving vaccine were expected to shed vaccine type virus (defined as positive for both NSP2 *and* VP6) at any point between 3 and 10 days after either dose 1 or dose 2 of RV1. The proportion of nearest aged siblings who would shed vaccine type virus was not known. The sample size calculations for a range of possible assumptions are shown in Table 8.2.

Table 8.2. Estimated sample size inflated for proportion of infants and nearest aged siblings shedding vaccine type virus.

	Proportion of nearest age siblings shedding*				
% infants Shedding*	**0.10**	**0.20**	**0.30**	**0.40**	**0.50**
40%	225	113	75	56	45
50%	180	90	60	45	36
60%	150	75	50	38	30
70%	129	64	43	32	26
80%	113	56	38	28	23

*where shedding is defined as a sample positive for vaccine type virus (on both NSP2 and VP6 assays) after dose 1 or dose 2 of vaccine. Numbers in table refer to number of nearest-aged siblings. Shaded area shows achievable targets with a sample size of 60.

A sample size of 60 nearest aged-siblings was selected. This corresponded with the lower limit of expected shedding in the vaccinated infant, and was sufficient to detect a range of proportions within the limits of what was pragmatic and feasible. Circumstances which would be detected with 80% power and 5% significance level by a sample size of 60 pairs are shaded in grey in the above tables. To explore risk factors for transmission each household member was invited to participate in the study. Each household was assumed to have a median of 5 household members in line with contemporaneous data from the diarrhoeal surveillance platform. Assuming a 25% loss to follow-up/drop-out rate, 75 households were planned to be recruited to achieve 60 completed households.

8.2.7 Laboratory procedures

All stool specimens were tested for rotavirus using VP6 qRT-PCR and with a vaccine virus specific qRT-PCR (NSP2) using the procedures outlined in Chapter 2 (section 2.3.4.6, page 93). Samples were considered positive for vaccine type virus if both NSP2 and

confirmatory VP6 assays were positive. Samples positive on NSP2 and negative on VP6 were classified as negative and samples positive on VP6 but negative for NSP2 were assumed to represent wild-type rotavirus. Data on HIV status and viral shedding density in the vaccinated infant were available from the RotaRITE:RI study.

8.2.8 Statistical analysis

To generate descriptive statistics, distributions of continuous variables were examined and categorical variables were tabulated. Missing observations were excluded from analysis. Chi squared tests were used to compare independent categorical variables and McNemar's tests were used to compare paired proportions. Two-sided t-tests were used to compare independent means of normally distributed data and rank sum tests were used to compare non-normally distributed data. Sign-rank tests were used to compare paired medians.

8.2.9 Managing specific variables

Questions regarding whether or not the household contact was responsible for changing the index child's nappy, and whether or not the contact was the primary care giver, were asked of adults or children over 5 years of age. Children under 5 years completed a separate form where these questions were not included. For analysis purposes it was assumed that children under 5 years were not primary care givers or responsible for changing the nappy, and the variables were recoded to reflect this.

Wealth

A proxy variable for relative wealth was constructed as described in Chapter 5 (section 5.2, page 154).

Shedding

Shedding in vaccine recipients was defined as vaccine virus shedding at any point between 3 and 10 days after either dose 1 or dose 2 of RV1, where vaccine virus shedding was defined as detectable vaccine type virus on NSP2 and on confirmatory qRT-PCR for VP6. Samples collected less than 3 days after vaccine receipt were excluded as any vaccine virus present at this time was considered likely to represent gut transit, rather than viral replication (131,370).

8.2.10 Ethics

This study was approved as an amendment to the RotaRITE:TE study by the University of Liverpool Research Ethics committee (000757), and the Malawi College of Medicine Research Ethics Committee (P.09/14/1623).

8.3 Results

8.3.1 Description of cohort.

Horizontal Transmission study recruitment took place between the 25th of April 2016 and the 8th of August 2016. Of 72 households who consented to take part in the study, 3 withdrew, leaving 69 households with follow up data (Fig. 8.2). These 69 households consisted of 287 household members, of whom 220 (76.7%) consented to take part in the study; 69 vaccinated infants and 151 contacts.

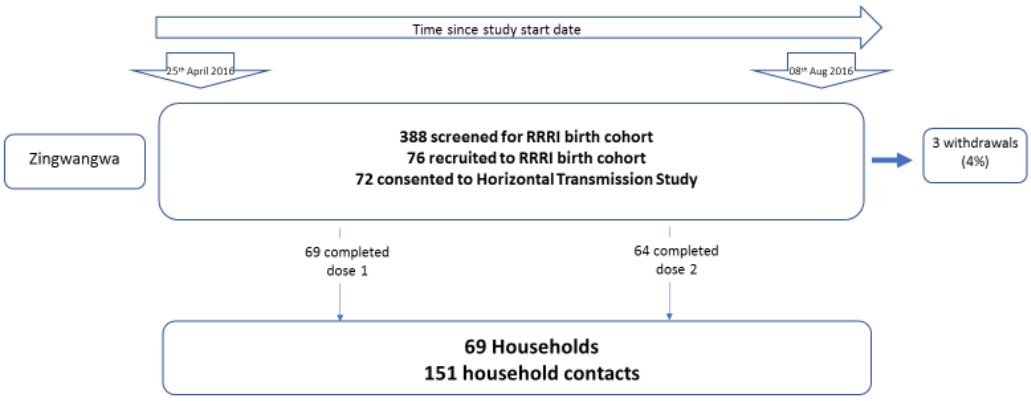


Figure 8.2. Overview of Horizontal Transmission Study recruitment

8.3.2 Description of vaccinated infants

Of the 69 infants who participated in the study, 64 had complete data on shedding post vaccination. Characteristics of the 69 recruited infants are summarised in Table 8.3. Median age at recruitment was 6.1 weeks (range 5.1 to 8.7 weeks). All were breastfed. A total of 12(17%) infants were born to mothers with HIV infection; 11 of these were successfully followed up to obtain HIV DNA PCR results, of which 5 were determined to be HIV-uninfected and in 6 infants the family did not yet know the HIV DNA PCR result. No infants reported diarrhoea or vomiting at the time of recruitment. Household size was a median of 4 (IQR 3,5). Food insecurity was common, with 32% of households reporting

difficulties obtaining the food they need in the last month, and 19% of adults skipping a meal in the last 2 weeks to ensure there was adequate food for the household. Median wealth was 2.5 (IQR 2.1, 2.9), which is consistent with findings of other cohorts in this study (Chapter 5, section 5.3, page 169).

Table 8.3. Characteristics of vaccinated infants

Co-variate	Summary statistic	Missing
Age at recruitment in weeks (IQR)	6.1 (6.0-6.4)	0/69
Male sex (%)	29/69 (42.0)	0/69
Diarrhoea (%)	0/69 (0.0)	0/69
Vomiting (%)	0/69 (0.0)	0/69
HIV (%)		
Exposed	12/69 (17.4)	0/69
Infected*	0/5 (0.0)	6/11
Anthropometry. Mean (SD)		
Weight for height Z score	0.72 (1.24)	2/69
Weight for age Z score	-0.48 (1.10)	0/69
Height for age Z score	-1.09 (1.25)	1/68
Mid upper arm circumference	12.5 (11.5, 13)	0/69
Household size (IQR)	4 (3,5)	0/69
Premature (%)	4/68 (5.9)	1/68
Birth weight (%)	3 (2.7, 3.3)	0/69
Ever Breastfed (%)	69/69 (100.0)	0/69
Additional child <1 (%)		
0	69/69 (100.0)	0/69
Additional children < 5 (%)		
0	46 (66.67)	
1	22 (31.88)	
3	1 (1.45)	0/69
Electricity at home		
Yes (%)	37/69 (53.6)	0/69
Shared toilet		
Yes (%)	54/69 (78.3)	0/69
Typically, how long does it take for household to access water? (%)		
Instant	9/69 (13.0)	
<5 mins	21/69 (30.4)	
5-30 mins	36/69 (52.2)	
30 mins-1hr	2/69 (2.9)	
>1hr	1/69 (1.5)	0/69
What is the typical source of domestic water (%)		
Borehole	4/69 (5.8)	
Covered well	1/69 (1.5)	
Open well	0/69 (20.3)	
Tap to house	14/69 (39.1)	
Shared tap compound	27/69 (39.1)	
Shared tap village	23/69(33.3)	0/69
Number of adults in the house with a regular salary		
0 (%)	18/69 (26.1)	
1	43/69 (62.3)	
2	7/69 (10.1)	
3	1/69 (1.5)	0/69
Problems getting food in the last month? (%)		

Never	45/69 (65.2)	
Sometimes	22/69 (31.9)	
Often	2/69 (2.9)	0/69
Has an adult skipped a meal in the past 2 weeks?		
Yes (%)	13/69 (18.8)	
Wealth indicator (IQR)	2.5 (2.1, 2.9) **	1/68

* Of 12 HIV exposed infants 11 were successfully traced for HIV PCR results. Of those, 5 were confirmed uninfected and 6 did not know their status/had not received their result.
**Where wealth is a composite variable compiled as described in chapter 5 (section 5.2, page 154).

Of the 64 vaccinated infants with complete shedding data, 45(70%) shed vaccine virus at any point (Table 8.4). The proportion of infants shedding was slightly higher post dose two than dose one (29/69 [42.0%] vs 34/64 [53.1%]) but this was not significant at the 5% level (Mcnemar's p value 0.150). The proportion of infants shedding was reasonably constant across days 4-10 following each dose of vaccine, with a trend towards a greater frequency of shedding at days 4-8 (Table 8.5). Shedding was low level, with peak shedding following vaccine dose one of median Ct 32.2(IQR 29.9-35.0) and 33.1(IQR 30.5-35.5) following dose two. There was no significant difference in median Ct value between doses (sign-rank p 0.9750) (Table 8.4). Over 20% of infants shed vaccine virus after both doses (14/64, 21.9%).

Table 8.4 Shedding in infants and household contacts

	% shedding NSP2 at baseline	% shedding post d1	Median peak Ct value post d1 (IQR)	% shedding post d2	Median peak Ct value post d2 (IQR)	% with any shedding
Infant	1/34 (2.9)	29/69 (42.0)	32.2(29.9-35.0)	34/64 (53.1)	33.1(30.5-35.5)	45/64(70.3)
HH contact	0/123 (0.0)	0/138 (0.0)	-	2/122 (1.64)	-	2/151(1.3)

Table 8.5 Shedding by day post vaccine dose in infants

	Days post vaccine dose 1				Days post vaccine dose 2			
	4	6	8	10	4	6	8	10
No shedding (%)	15/58 (25.9)	18/61 (29.5)	15/56 (26.8)	12/65 (18.5)	20/57 (35.1)	21/60 (35.0)	20/57 (35.1)	17/55 (30.9)

8.3.3 Description of households

151 household contacts were recruited. Characteristics of household contacts are shown in Table 8.6. 46 (31%) were male. Age of contacts ranged from under 1 year to 41 years old (median 20.3, IQR 9.6, 29.35). 70/151 (46%) of recruits were mothers of the vaccinated infants and 51/151 (34%) were children who were relatives of the vaccinated

infant. Fathers made up 13% (20/151) of recruits. Of the 17 contacts aged under 5 years with available data 7 (41%) were completely vaccinated against rotavirus. The majority of household contacts were in close contact with the vaccinated infant with 109/151 (72%) sharing a bedroom, and 85/151 (56%) typically spending all day in the company of the child. Sanitation facilities were basic, with 93% (140/151) reporting that they typically use a simple pit latrine. No contacts reported diarrhoea or vomiting during follow up.

Pre-vaccine exposure samples were obtained from 123 contacts and none were positive for vaccine virus. Post-exposure samples were collected a median of 8 days (IQR 8,8) after sample 1 and 8 days after sample 2 (IQR 8,10). A total of 2/151 (1.3%) household contacts shed vaccine virus following exposure to a vaccinated infant; one sibling (age 3 years) and one mother (age 34 years)(Table 8.4). This was not significantly different from baseline (McNemar's P value 0.500). Both of the positive samples were collected after the second dose of vaccine administered to the infant. The mother was reported to be HIV negative, shared a bedroom with the vaccinated infant, spent the whole day with the child and was responsible for changing the infant's nappy. The sibling contact did not share a bedroom and spent an estimated half of the day in the company of the vaccinated infant. The sibling contact had not been tested for HIV. Of household members, 9 (6%) had detectable wild-type rotavirus in at least one stool sample. Wild type shedding was more common in those under 10 years (4/40 [10%]) than those aged over 10 years (5/111 [5%]), though this was not significant at the 5% level (X^2 p value 0.21). Due to the small number (two) of transmission episodes it was not possible to formally investigate risk factors for horizontal transmission.

Table 8.6 Characteristics of household contacts

Co-variate	Summary statistic	Missing
Age category in years (%)		
0-5	20/151 (13.3)	
5-10	20/151 (13.3)	
10-15	12/151 (8.0)	
15-30	70/151 (46.4)	
30-45	29/151 (19.2)	
45+	0/151 (0)	0/151
Male sex (%)	46/151 (30.5)	0/151
Diarrhoea (%)	0/151 (0)	0/151
Vomiting (%)	0/151 (0)	0/151
HIV		
Ever tested (%)	79/149 (53.0)	0/149
HIV Infected (%)	13/77 (16.9)	2/79
Relationship to child (%)		
Mother	70/151 (46.4)	
Father	20/151 (13.3)	
Grandmother	1/151 (0.7)	
Grandfather	0/151 (0)	
Other adult	9/151 (6.0)	
Child relative	51/151 (33.8)	
Child non-relative	0/151 (0)	0/151
Rotavirus vaccine doses in under 5s (%)		
0 doses	9/17 (52.9)	
1 doses	1/17 (5.9)	
2 doses	7/17 (41.2)	1/18
Sleeps in same room as vaccinated infant (%)	109/151 (72.2)	0/151
Sleeps in same bed as vaccinated infant (%)	91/151 (60.3)	0/151
Time spent in house (%)		
All day	85/151 (56.3)	
Half day	44/151 (29.1)	
Evening only	22/151 (14.6)	0/151
Time spent with child (%)		
All day	85/151 (56.3)	
Half day	44/151 (29.1)	
Evening only	22/151 (14.6)	0/151
Toilet type (%)		
Simple pit	140/151 (92.7)	
VIP	4/151 (2.7)	
Water toilet	7/151 (4.6)	0/151
Primary care giver for vaccinated infant (%)		
Yes	72/151 (47.7)	0/151
Responsible for changing nappy of vaccinated infant (%)		
Never	76/151 (50.3)	
Sometimes	5/151 (3.3)	
Often	0/151 (0)	
Always	70/151 (46.4)	0/151

8.4 Discussion

This study found a very low frequency of horizontal transmission of vaccine virus to household contacts of vaccinated infants in Malawi, a low income country in sub-Saharan Africa. In this setting, it seems unlikely that horizontal transmission of vaccine is a major contributing factor to rotavirus vaccine indirect effects. This is the first study to investigate rotavirus vaccine virus transmission in households in a low income setting, where immune response to vaccine, nutritional status, contact patterns and sanitation systems are very different to those in higher income countries.

This study has the major advantage of using qRT-PCR to detect vaccine virus shedding. The majority of previous studies have used EIA or cell culture, which is considerably less sensitive than qRT-PCR and provides an under-estimate of the degree of transmission. All positive results were confirmed with a second (VP6) qRT-PCR to ensure reliability. This is also the first study to describe transmission of rotavirus vaccine virus to all household contacts, enabling risk factors for transmission to be evaluated, should there have been enough events for meaningful analysis.

These findings corroborate the findings of a study conducted on infants in contact with RV1 vaccinated infants in a Japanese foster home which also used qRT-PCR to detect vaccine virus, and identified one possible episode of vaccine virus transmission in a contact who had previously received rotavirus vaccine. The numbers recruited were relatively small (4 vaccinated infants and 23 contact infants) however, and in contrast to this study did not include care givers or older children. The low frequency of transmission is however in contrast to a study by Rivera et al who conducted a randomised controlled trial (RCT) of RV1 transmission in twins in the Dominican republic(244). Transmission was defined using EIA, and the study found transmission rates of almost 20%, a quarter of whom sero-converted. Potential explanations for these differences include higher rates of shedding in vaccinated infants in the Dominican Republic, perhaps due to more robust immune response to vaccination, or differences in contact patterns or the age of the contact. Infants in the Japanese study had a median age of 9 months, and median age of contacts in our study was 20 years. In comparison twin siblings at the age of vaccination (1-3 months) are likely to have little pre-existing immunity to rotavirus, even compared to infants 9 months of age, and therefore may be more likely to become infected with vaccine virus. It is also possible that twins spend more time in close proximity with each other than infants in a foster home, or older relatives.

It is also intriguing that vaccine virus transmission differs so substantially from wild-type transmission in the same socio-cultural environment. The most likely explanation is a combination of presence of symptoms and viral load. Peak viral load was not associated with risk of transmission for infection or disease in our setting (Chapter 7), however in symptomatic children median cycle thresholds for the first sample after presentation were 19.1 (Chapter 6, section 6.3.1, page 195), compared to a cycle threshold of 32 with vaccine virus. It may be that differences in viral load are not associated with risk of transmission in symptomatic children because they all have very high viral loads, but that once viral loads become substantially lower transmission is less likely. This is corroborated by the fact that no new episodes of infection were identified in household contacts of symptomatic index children beyond the first two weeks after initial exposure, despite the fact that the majority of index children were still shedding at low levels (Chapter 6, section 6.3.4, page 203). The importance of symptoms in transmission is demonstrated by the association between symptom severity and transmission identified in this thesis (Chapter 7, section 7.3, page 214), and also by a study from Ecuador, which identified rotavirus infection in 55% of households of symptomatic index cases, 8% of household contacts of rotavirus EIA negative children with diarrhoea, and 2% of household contacts of healthy control children(106). None of the infants recruited into this horizontal transmission study reported symptoms.

This is the first study of rotavirus vaccine virus transmission conducted in a setting with high background HIV prevalence. This is relevant as there is some evidence that HIV infected children may shed rotavirus for longer following rotavirus infection, and children with severe immunodeficiencies have been shown to shed rotavirus, including vaccine virus, for extended periods of time(260,373). In this study prevalence of HIV infection in household contacts was 17%, consistent with the background prevalence of HIV in Blantyre, Malawi, estimated at 18.5% in 2010(374). None of the vaccinated infants had confirmed HIV infection, although 12 were known to be HIV exposed. The adult contact with detectable vaccine strain was reported to be HIV negative when last tested, and the child contact who shed vaccine virus had never been tested for HIV. With the caveat that this study was not designed or powered to formally investigate the relationship between HIV exposure in a household and risk of vaccine virus transmission, there is no evidence from this study that vaccine virus transmission is significant with populations with a high background prevalence of HIV.

8.4.1 Limitations

It is notable that males, and particularly fathers, are under-represented in household contacts to a greater extent than was observed in the primary household transmission study (13% of recruits vs 19% in the primary study, chapter 5, section 5.3, page 169). This is likely to represent the method of recruitment for this study, which was predominantly based at the health centre with household contacts bringing samples to the health centre, in contrast to the primary study where the field team visited and recruited from the home. The primary transmission study also often recruited children who were admitted to hospital, in which case extended family members including fathers visited, facilitating contact. Although fathers are under-represented, this is unlikely to have affected the validity of results as we have seen so little transmission in mothers, who typically have much more direct contact with the vaccinated infant, or in young children who are likely to be more susceptible to rotavirus infection.

Data for this study were collected from April 2016 through August 2016 which is not a complete year. The start of recruitment was delayed due to delays in ethical approval, and recruitment had to stop in August because the RotaRITE:RI study completed recruitment. Wild-type rotavirus shows some seasonality in Malawi, with the peak of disease occurring between May and October(34). Recruiting for only part of a year could mean that the prevalence of wild-type rotavirus in household contacts is misleading, but would be unlikely to significantly affect rates of transmission of vaccine virus.

Single stool samples from contacts were collected at either day 8 or day 10 after vaccination in the infant and it is possible that some transient shedding episodes were not detected by this method. Serial stool samples in contacts would have been a more robust way to ensure all episodes were captured, but unfortunately that was not possible due to funding and logistics. However shedding of vaccine virus occurred early in vaccinated infants, with a similar proportion of infants observed to be shedding at day 4 as at day 10; qRT-PCR was used to detect vaccine virus which is highly sensitive even to very low level viral shedding and the study findings are corroborated by those from the study in Japan which did conduct serial sampling(368). These findings may represent a minimum estimate of horizontal transmission, but it seems unlikely that the true frequency should differ substantially from this.

Conclusions, implications and further studies

This study identified very little horizontal transmission of vaccine virus to household contacts in Malawi, despite high background HIV prevalence, crowded living conditions and poor sanitation, and as such horizontal transmission of vaccine virus seems unlikely to be a major contributing factor to indirect effects in this setting. This does not however preclude the presence of substantial indirect effects through reduction in transmission of wild-type rotavirus, and studies which build on the work in this thesis and attempt to quantify these effects are needed.

In order to understand the implications of horizontal transmission and to detect transmission episodes not identified using faecal shedding, testing sero-response in household contacts would be very useful. This was not possible in the context of our study due to cost and logistics, and because blood collection in the community is subject to complex social implications which need negotiating, however future studies should consider incorporating this into study design. Any future studies of horizontal transmission should also consider collecting serial samples from contacts to minimise the risk of under-detecting shedding episodes.

9. Discussion and Conclusions

9.1 Overview

Global rotavirus vaccine introduction has had an enormous public health impact, and it is a remarkable achievement that the trajectory from first discovery of rotavirus in the 1970s, description of the burden of disease, vaccine development, global vaccine introduction and post-implementation evaluation studies have occurred over less than half a century. However despite these successes, and even in the context of highly successful vaccine campaigns and higher than expected vaccine effectiveness estimates, rotavirus remains the commonest cause of admitted gastroenteritis in children from some sub-Saharan African settings(329). In order to maximally protect the most vulnerable children from rotavirus and inform ongoing vaccine policy, it is necessary to understand mechanisms for the reduced vaccine effectiveness in low income settings; to characterise the residual burden of disease; to describe rotavirus epidemiology in vaccinated populations and to evaluate the total population level impact of the vaccine, including any indirect effect(215).

Very little is known about rotavirus transmission in LICs, yet it is crucial to all of the unanswered questions above. High force of infection may contribute to the reduced vaccine effectiveness observed in LICs and the age distribution of rotavirus infection and disease is likely to relate to transmission dynamics. In terms of evaluating population level impact, vaccine mediated reductions in rotavirus transmission via reduced frequency of infectious contacts, reduction in infectiousness of symptomatic cases or transmission of vaccine virus to unvaccinated contacts have the potential to increase protection afforded by vaccine to vaccinated infants and protect members of the population that have not themselves been vaccinated. Such vaccine indirect effects can be sufficient to transition a vaccine programme from cost effective to cost saving(229), but there are a paucity of data from LICs where these effects are most crucial.

This thesis set out to address some of these questions. The results are divided into two sections. Section A consists of two chapters and focuses on rotavirus transmission at a population level. The first chapter explores a novel and pragmatic technique to estimate rotavirus force of infection in different populations which has the potential to increase our understanding of how transmission dynamics may relate to global disparities in vaccine performance. The second uses surveillance data to quantify rotavirus vaccine

indirect effects for the first time in a LIC, and to describe the changing epidemiology of rotavirus in a sentinel surveillance site following vaccine introduction.

Having identified evidence of rotavirus vaccine indirect effects in a Malawian population, and differences in patterns of indirect effects compared to those from in higher income settings, Section B of the results focusses on rotavirus transmission at a household level to understand mechanisms driving rotavirus vaccine indirect effects in a LIC. Section B is further divided into two main questions. Firstly whether rotavirus vaccine has potential to reduce the infectiousness of an index case. This is addressed in stages; by defining SAR in a LIC in sub-Saharan Africa, investigating whether disease severity is associated with viral load in index children, and investigating whether disease severity and viral load are associated with risk of transmission at the household level. Secondly, it asks whether horizontal transmission of vaccine virus and subsequent generation of herd immunity is likely to contribute to rotavirus vaccine indirect effects in a Malawian population.

This current chapter summarises the findings of the thesis, and outlines implications for vaccine policy and future work which may arise.

9.2 Summary of findings

Section A

By utilising a novel method of analysing rotavirus sero-prevalence data, Chapter 3 demonstrates substantial differences in timing of first exposure to rotavirus in infants from two different unvaccinated low-income populations, with infants from Vellore, Southern India exposed to rotavirus at an earlier age than children from Karonga, Northern Malawi. Reasons for this difference are not known, but may include differences in population density, poverty levels, or prevalence of co-morbidities such as malnutrition. High force of rotavirus infection has been cited as one possible explanatory factor for low rotavirus vaccine effectiveness in LICs because of high maternal antibody levels which could interfere with vaccine immunogenicity in infants(142,143), or because high frequency of natural exposure in unvaccinated control groups impacts on measurement of rotavirus vaccine effects(145). Describing heterogenicity in force of infection between populations will increase our understanding of the relationship between force of infection and vaccine response, and mixture models may be a useful and pragmatic analytical technique to support this.

Using data from 5 years of diarrhoeal surveillance at QECH, Chapter 4 describes a consistent decline in the prevalence of rotavirus attributable gastroenteritis requiring hospitalisation in Blantyre, Malawi, following programmatic vaccine introduction in 2012. It also identifies an additional reduction in incidence of hospitalised rotavirus gastroenteritis of any severity of 9-24% in infants beyond that predicted based on vaccine efficacy and vaccine coverage. This is the first quantifiable evidence of rotavirus vaccine indirect effect from a LIC. However in contrast to findings from higher income settings indirect effects were not identified in infants with severe disease or children over 12 months of age(221). In addition, the observed reduction in the prevalence of rotavirus attributable gastroenteritis was less pronounced in children aged 1-2 years of age compared to infants under one year; the proportion of rotavirus gastroenteritis occurring in children aged 12-24 months increased from 18 to 38% following vaccine introduction and the point estimate for vaccine effectiveness was substantially lower in children aged 12-24 than those under 12 months of life. Despite high vaccine coverage and encouraging vaccine effectiveness estimates in infants, rotavirus remains a major cause of hospitalised gastroenteritis in Blantyre, responsible for over one quarter of all admissions for gastroenteritis.

Section B

Could rotavirus vaccine reduce the infectiousness of a vaccinated index case?

Rates of transmission for rotavirus infection were very high in household members exposed to a symptomatic index child, but rates of disease were much lower. Rotavirus disease was commoner in household contacts under 5 years, but transmission rates for infection did not vary according to the age of the household contact. In this population repeated exposure appears to result in immunity to disease but not infection. Rates of infection were comparable to those identified by a study in Ecuador which also used qRT-PCR(106), but disease rates were notably lower. One explanation for this could be high background infection rates in Malawi contributing to maintenance of immunity against clinical disease. This hypothesis is supported by the high frequency (27%) of asymptomatic rotavirus infection described in control households without history of recent exposure to a child with diarrhoea.

Index children with clinical rotavirus gastroenteritis shed large quantities of rotavirus in their stool, corroborating previous findings from India. Shedding density declined quickly, but in some children ongoing low level shedding persisted for weeks after symptom onset.

Children with more severe disease shed more virus than children with milder disease, suggesting that interventions to reduce disease severity, for example vaccine, could reduce viral shedding density in index children and therefore transmission. Viral load in household contacts declined with time from onset of symptoms in the index child, supporting the hypothesis that household contacts are infected from a symptomatic infant in the house.

Increasing disease severity in the index child was associated with an increased risk of both rotavirus infection and disease in household contacts. Contrary to what was hypothesised this did not appear to be mediated through a reduction in viral shedding density, which showed no association with transmission of infection or disease at the univariate or multivariate level. Transmission of infection showed no relationship with the age of household contact, but risk of disease was significantly higher in children under 5 years of age. Contact with the index child was a risk factor for infection, with mothers at significantly greater risk of infection than any other household relative. The association between disease severity in the index child and risk of transmission for both infection and disease suggests that vaccination has the potential to reduce the infectiousness of a child with rotavirus gastroenteritis even in the event of clinical vaccine failure, as rotavirus vaccine provides incremental protection against severe disease. As a result of the successful vaccine programme and high vaccine coverage in Blantyre we were unable to identify enough unvaccinated children to directly investigate the effect of vaccine on rotavirus transmission.

Could horizontal transmission of monovalent human rotavirus vaccine virus (Rotarix) to household contacts contribute to rotavirus vaccine indirect effects in Malawi?

Very low rates of horizontal transmission of vaccine type virus were observed in household in Blantyre, Malawi, with 2/151 (1.3%) of household contacts of vaccinated infants found to be shedding vaccine virus. This was despite high frequency of low level shedding in vaccinated infants and a high background prevalence of HIV exposure. Horizontal transmission of vaccine virus does not appear likely to be a major mechanism underpinning the production rotavirus vaccine indirect effects in Malawi.

9.3 Implications

Force of infection in low income countries

Rotavirus remains a major cause of diarrhoeal disease requiring hospitalisation in Malawi. This has ongoing implications for health care provision and resource management, as well as substantial social and economic ramifications for the community. It is possible that as force of infection drops following vaccine introduction rotavirus disease burden in LIC will to decline, but if disease burden remains high with continued surveillance and sustained high vaccine coverage, then a change to vaccine strategy either with modification to scheduling for current vaccines or with novel candidate vaccines may be required(294). Understanding patterns of force of infection in different settings, for example with serological data, could inform decisions around optimal vaccine scheduling. For example a very high burden of early disease may require a neonatal dose of vaccine, or alternatively a delayed vaccine dose once the influence of maternal immunity has waned.

High force of infection may also influence the measures of effect currently used for vaccine studies, such that studies conducted in high disease burden settings are not directly comparable with low income settings. As argued by Gomes at al(145), in settings where force of infection is high vaccine efficacy may represent cumulative efficacy against several episodes of disease and per-event estimates of efficacy could result in estimates which are more comparable with lower burden settings. Similarly, because of high frequency of exposure to rotavirus in unvaccinated children and acquisition of natural immunity, high force of infection could in part explain the reduced vaccine effectiveness in the second year of life observed in Malawi and why indirect effects appear less substantial in older children than reported from higher income settings (Chapter 4). If the extent of rotavirus vaccine indirect effect described in Chapter 4 is accurate and vaccine indirect effects are indeed limited to infants, then indirect effects may play a lesser role in population level vaccine impact in a Malawian population than hoped. However, if this is in part a function of high force of infection then vaccine indirect effects and indeed observed vaccine effectiveness may actually *increase* over time as population level incidence drops. Additional vaccine doses may then become less urgent.

Rotavirus transmission in Malawi

This thesis provides a first step in understanding the complexity of rotavirus transmission in low income settings and considering how vaccine may impact on this. Very high levels of rotavirus infection were observed in households following exposure to a symptomatic index case, but much lower levels of disease were seen. It may be that regular exposure to rotavirus in late child- and adult-hood results in maintenance of immunity against disease, and it is possible disease attack rates will increase over time if population level transmission drops as a result of vaccine. A high frequency of asymptomatic shedding of rotavirus was also observed in households which had not recently been exposed to a symptomatic index case. This high frequency of low level rotavirus shedding may at least partly explain the great diversity of rotavirus strains observed in Malawi and other low income settings. The frequency of asymptomatic shedding and the fact that there was no observed reduction in asymptomatic shedding with age calls in to question whether individuals in Malawi develop immunity to rotavirus infection. The contribution of this asymptomatic shedding to ongoing transmission is as yet unknown.

Mechanisms of rotavirus vaccine indirect effects

The positive association between disease severity and household transmission of infection and disease provides evidence to support the hypothesis that vaccine mediated reductions in disease severity could reduce household transmission in the event of vaccine failure. Given a vaccine effectiveness of approximately 60% in Malawi, this could have a substantial impact on transmission at the population level. From this study, it seems that horizontal transmission of vaccine virus, or generation of herd immunity, is not a major contributor to rotavirus vaccine indirect effects in our setting. That horizontal transmission of vaccine virus is so infrequent an occurrence, even in a population with high HIV and malnutrition prevalence, should reduce any anxiety about the risk of harmful transmission to children or adults with immunocompromise

9.4 Further studies and work building on this thesis

The work in this thesis expands our knowledge of rotavirus transmission in low income settings and how this may influence vaccine effects; however several additional research questions arise from the work described here. These are outlined below.

1. Is pre-existing immunity against rotavirus associated with disease severity, viral shedding density and risk of transmission?

This thesis has demonstrated a positive association between disease severity and transmission, and between viral load and disease severity, but the role of pre-existing immunity to rotavirus in these phenomena is not known. If higher titres of anti-rotavirus IgA at presentation at baseline are associated with reduced disease severity and reduced risk of transmission to household contacts this would add weight to the hypothesis that vaccine could reduce infectiousness. In order to address this question serum samples were collected from index children at the time of presentation for measurement of anti-Rotavirus IgA titres. These data are not yet available, but will form the basis of future analyses to address the above question.

2. Is high force of infection associated with reduced vaccine effectiveness?

This question has two strands. Firstly, could high force of infection have a causative role in sub-optimal vaccine effectiveness, either as a result of reduced vaccine immunogenicity mediated by high maternal anti-bodies, or because of a high burden of disease before infants have been adequately vaccinated(294). If this is the case then interventions such as changes to dosing schedules or novel candidate vaccines could have an important role to play in maximising protection. A clearer understanding of different patterns of transmission epidemiology in the first year of life is then important to understand how to target interventions. Sero-prevalence data of rotavirus in infants has the potential to improve understanding of incidence patterns in different populations, and if this is combined with vaccine efficacy or effectiveness data from the same populations has potential to provide insight into the relationship between force of infection and vaccine performance. Sero-prevalence data from multiple different counties and socio-economic settings exists from vaccine pre-licensure clinical trials. These data have the major advantage that the serology was conducted in a standardised way using the same assay, and is therefore comparable and suitable for meta-analyses. Mixture models could be a pragmatic and effective means of estimating force of infection from such sero-prevalence data.

In terms of potential interventions, immunogenicity trials of different dosing strategies have been conducted and describe increased immunogenicity with either a delayed additional dose (14 weeks)(310), or a booster dose (9 months)(375), and neonatal dosing strategies of a tetravalent rotavirus vaccine have been shown to be immunogenic and demonstrate good efficacy against rotavirus gastroenteritis of any severity (60%) (311). A Phase 2b trial of a novel candidate vaccine based on a neonatal strain (RV3), which has

shown promising results from immunogenicity studies in New Zealand(131), is planned to be undertaken in Malawi.

Secondly, could force of infection effect the *measurement* of rotavirus vaccine indirect effects and rotavirus vaccine effectiveness: in other words could high levels of natural exposure and subsequent immunity in older unvaccinated children explain some of the observed disparity between LIC and HIC. A low burden of disease in older children due to pre-existing immunity could explain the absence of identifiable indirect effects in children over 12 months of age, and natural exposure to wild-type disease in unvaccinated children could result in comparison groups becoming too similar, and result in reduced vaccine effectiveness estimates. It seems unlikely to be the only explanatory factor explaining reduced vaccine performance in LIC given the sub-optimal immunogenicity also observed in such settings from clinical trials(298). If, however, such a phenomenon does make a contribution it maybe that observed vaccine effectiveness and indirect effects to older age groups increase as transmission decreases as a result of vaccine introduction. Careful ongoing surveillance is necessary to evaluate this, to identify at-risk groups, and enable observation of trends in rotavirus prevalence with time. Given the success of the vaccine campaign and the low frequency of unvaccinated children further direct estimates of VE are not possible and ongoing evaluation will requires more complex analytical techniques and mathematical modelling. Ongoing sentinel surveillance at QECH will inform mathematical models of rotavirus vaccine indirect effects. These analyses will be invaluable in understanding the effect on changes in transmission on vaccine effects in our setting.

3. What is the role of asymptomatic infection in immunity against rotavirus disease?

Asymptomatic rotavirus shedding in the community in Blantyre, is extremely common but its role in the maintenance of immunity is not clear. The high frequency of asymptomatic infection may act as a “booster” to the immune system and provide protection against rotavirus disease. This could explain why we see so little disease in household contacts of symptomatic index cases. Alternatively the high frequency of asymptomatic rotavirus shedding could reflect an inability to mount a complete immune response to rotavirus infection. If this is different to patterns in high income settings it may add insight into observed reduced vaccine immunogenicity. It will only be possible to evaluate this if similar household studies with comparable molecular techniques used to detect rotavirus are conducted in higher income settings. There is some evidence that children from low

income settings shed rotavirus for longer following a symptomatic episode than children from high income settings (254,255)although as a result of heterogenicities between studies this requires further validation. To investigate this, cohort studies which follow up children with symptomatic rotavirus for an extended period of time are needed in both low and high income settings. If differences in clearance of rotavirus infection are confirmed this could add information to our growing understanding of the mucosal immune response to rotavirus – perhaps delayed clearance of rotavirus reflects suboptimal mucosal immunity in low income settings – and further inform understanding of reduced vaccine immunogenicity in LIC.

4. What is the magnitude of rotavirus vaccine indirect effects in Blantyre, Malawi

This study identified some evidence of indirect effects of rotavirus vaccine in infants following rotavirus vaccine introduction, but not in children aged over 12 months of age. This is intriguing, as it is contrary to findings in higher income settings(221). There are reasonable epidemiological hypotheses which could explain this, for example a low burden of symptomatic disease in older children due to frequent re-exposure, but the findings are limited by a short duration of pre-vaccine introduction surveillance data, and ongoing evaluation is impossible due to high vaccine coverage. Addressing this question therefore requires more complex mathematical models, and this is being undertaken with collaborators at Yale School of Public Health.

5. What is the vaccine effectiveness on infectiousness of an index child to household contacts?

It was not possible to directly estimate the effect of vaccine on infectiousness because of the high vaccine coverage in Blantyre. However it may be possible to model vaccine effectiveness on infectiousness using data on the relationship between disease severity and transmission demonstrated in this thesis and existing estimates of vaccine effectiveness for different degrees of disease severity. Strategies to do this are currently under discussion. Such an estimate would provide a quantitative value for reduction in household rotavirus transmission mediated via reduced infectiousness of an index child and contribute to evaluations of population level rotavirus vaccine impact.

All of the above questions feed into one, over-arching question which extends beyond the scope of this thesis:

Is additional intervention required to improve protection of Malawian children against rotavirus disease?

The answer to this question is complex, and will require assimilation of data from multiple different locations and sources. It is possible that disease burden will continue to decline without intervention as a result of vaccine impact on transmission, but it is also possible that additional changes to the dosing schedule, or novel vaccines, could play an important role in reducing the ongoing burden of morbidity and mortality caused by rotavirus. Any policy decision will require careful weighing of further data, including assessment of ongoing disease burden informed by surveillance data, accurate assessment of vaccine effects incorporating indirect effects, field evaluations of possible interventions and predictive modelling studies.

9.5 Conclusions

Rotavirus remains an important cause of hospitalised diarrhoeal disease in children in Blantyre, Malawi, despite high vaccine coverage and encouraging vaccine effectiveness estimates. An additional, indirect effect of rotavirus vaccine is seen, but to a lesser magnitude than described from higher income settings. Rotavirus is remarkably transmissible, with SAR for infection within households of over 65%. Reducing disease severity in the index child reduces rates of transmission to household contacts, supporting the hypothesis that vaccination could reduce infectiousness of index children who fail vaccine. Horizontal transmission of vaccine virus to generate herd immunity is unlikely to be a major contributor to rotavirus vaccine indirect effects in our setting. A detailed understanding of patterns and drivers of rotavirus transmission is essential to understanding disparities in vaccine performance between different populations, evaluating the total impact of the vaccine and making policy decisions to best protect children in the world's poorest countries from rotavirus disease.

10. Appendices

Table A1. Search terms for literature review of rotavirus epidemiology

Rotavirus & middle income
Rotavirus & low & income
Rotavirus & central & Europe
Rotavirus & middle & east
Rotavirus & North Africa
Rotavirus & South & America
Rotavirus & Latin & America
Rotavirus & Cental & America
Rotavirus & Asia
Rotavirus & Africa
Rotavirus & Malawi
Rotavirus & Africa & asymptomatic
Rotavirus & asymptomatic
Rotavirus & mortality
Rotavirus & efficacy
Rotavirus & effectiveness
Rotavirus & vaccine & impact
Rotavirus & vaccine & effect
Rotavirus & vaccine & indirect
Rotavirus & transmission
Rotavirus & family
Rotavirus & families
Rotavirus & household

Table A2. Relative risk of rotavirus detection in children admitted to QECH with gastroenteritis for annual time periods since vaccine introduction

	RV** negative	RV positive	Total	RR (95% CI[†])*
Time period				
Pre-vaccine (Jan'12-Oct'12)	202 (55.49)	162 (44.51)	364	1 (ref)
Nov'12-Oct'13	365 (64.49)	201 (35.51)	566	0.81 (0.69, 0.94)
Nov'13-Oct'14	322 (74.71)	109 (25.29)	431	0.58 (0.48, 0.71)
Nov'17-Oct'15	382 (72.62)	144 (27.38)	526	0.64 (0.54, 0.77)
Nov '15-Jun'16	173 (73.93)	61 (26.07)	234	0.60 (0.47, 0.76)
Total	1,444 (68.08)	677 (31.92)	2,121	

Table A3. Comparison of characteristics of index children for children recruited in QECH and children recruited from health centres.

	QECH Summary statistic	Missing data	Health centre Summary statistic	Missing data	P value†
Age (median and IQR)	11.0(8.7,14.6)	0/113	12.8(9.0,16.0)	0/83	0.35**
Sex (male) (%)	68/113 (60.2)	0/113	40/83(48.2)	0/83	0.096
Diarrhoea (%)	113/113 (100)	0/113	83/83 (100)	0/83	-
Duration (days)		0/113		0/83	
1-3 (%)	96/113 (85.0)		77/38 (92.8)		
5	9/113 (8.0)		3/83 (3.6)		
≥6	8/113 (7.1)		3/83 (3.6)		0.242
Episodes(n)***		0/113		0/83	
1-4 (%)	12/113 (12.2)		12/83 (14.5)		
5	48/113 (43.9)		36/83 (45.8)		
≥6	53/113 (43.9)		33/83 (39.8)		0.535
Vomiting (%)	105/113 (92.9)	0/113	77/83 (92.8)	0/83	0.968
Duration (days)		0/105		0/77	
1 (%)	8/105 (7.6)		15/77(19.5)		
2	19/105 (18.1)		40/77(52.0)		
≥3	78/105 (74.3)		22/77 (28.6)		<0.001
Frequency (n)		0/105		0/77	
<5 (%)	67/105 (63.8)		56/77 (72.7)		
≥5	38/105 (36.2)		21/77 (27.3		0.204
HIV					
Exposed (%)	17/113 (15.0)	0/113	8/83 (9.6)	0/83	0.262
Infected (%)	2/42 (4.8)	71/113*	0/16 (0)	67/83	0.374
Completed rotavirus vaccination (%)					
Vaccinated (2 doses)	111/113 (98.2)	0/113	83/83 (100)	0/83	0.223
Admitted (%)					
Yes	111/113 (98.2)	0/113	0/83 (0)	0/83	<0.001
Vesikari score (IQR)	15 (14, 16)	3/113	12 (10,14)	0/83	<0.001*
Temperature (rectal, ^{o}C)					
37.1-38.4 (%)	46/111 (41.4)		46/82 (56.1)		
38.5-38.9	28/111 (25.2)		20/82 (24.4)		
≥39.0	37/111 (33.3)	2/113	16/82 (19.5)	1/83	
Thirst (%)					
No thirst	8/113 (7.1)		24/83 (28.9)		
Thirsty	86/113 (76.1)		55/83 (66.3)		
Drinks poorly	19/113 (16.8)	0/113	4/83 (4.8)	0/83	<0.001
Skin pinch (%)					
Normal	8/113 (7.1)		48/83 (57.8)		
Goes back slowly	70/113 (62.0)		34/83 (41.0)		
Goes back very slowly	35/113 (31.0)	0/113	1/83 (1.2)	0/83	<0.001
General Appearance (%)					
Well, alert	36/113 (31.9)		58/83 (69.9)		
Restless	58/113 (51.3)		25/83 (30.1)		
Unconscious	19/113 (16.8)	0/113	0/83 (0)	0/83	<0.001
Dehydration (%)					

None	4/113 (3.5)		22/83 (26.5)		
Some (5%)	68/113 (60.2)		56/83 (67.5)		
Severe (10%)	41/113 (36.3)	0/113	5/83 (6.0)	0/83	<0.001
IV fluids (%)					
Yes	58/113 (51.3)	0/113	0/83 (100.0)	0/83	<0.001
Oral fluids (%)					
Yes	112/113 (99.2)	0/113	73/83 (88.0)	0/83	0.001
Outcome (%)					
Home	111/113 (98.2)		83/83		-
Died	2/113 (1.8)	0/113	0/83	0/83	0.223
Anthropometry, mean (SD)					
Adjusted WHZ	-1.00(1.5)	1/113	-0.34 (1.7)	0/83	0.005*
Adjusted WAZ	-0.55(1.2)	1/113	-0.14 (1.1)	0/83	0.061*
Adjusted HAZ	-0.41 (2.5)	5/113	-0.50(2.4)	0/83	0.708*
MUAC	13.2 (1.3)	0/113	13.5 (1.2)	1/83	0.090*
SAM	17/113 (15.0)	0/113	6/81(7.4)	2/81	0.105
Previous diarrhoeal admission (%)	11/113 (9.7)	0/113	4/83 (4.8)	0/83	0.201
Previous diarrhoeal presentation (%)	69/113 (61.1)	0/113	22/83(26.5)	0/83	<0.001
Premature (%)	4/113 (3.5)	0/113	3/53 (3.6)	0/83	0.978
Birth weight, mean (SD)	2.99 (0.64)	12/113	2.92(0.63)	4/83	0.381*
Ever Breastfed (%)	112/113 (99.1)	0/113	0/83 (0.0)	0/83	0.390
Diet includes food other than breast milk (%)	109/113 (96.5)	0/113	81/83(97.6)	0/83	0.650

†P values are X^2 p values for differences in proportions between case children recruited at QECH or health centres unless otherwise specified. *2 sided independent ttest **rank sum test

Table A4. Comparison of index children who completed study to index children withdrawn from study

	Index children who completed		Index children who withdrew		
	Summary statistic	Missing data	Summary statistic	Missing data	P
Age (Median and IQR)	11.5 (8.8, 15.2)	0/196	11.0 (8.3, 13.9)	0/59	0.215
Sex (male) (%)	108/196 (55.1)	0/196	41/59(69.5)	0/59	0.049
Diarrhoea	196/196	0/197	59/59 (100)	0/59	-
Duration (days)		0/196			
1-3	173/196 (88.3)		48/59 (82.4)	0/59	
5	12/196 (6.1)		7/59 (11.9)	0/59	
≥6	11/196 (5.6)		4/59 (6.8)	0/59	0.307
Max per day (n)		0/196			
1-4	24/196 (12.2)		13/59 (22.0)	0/59	
5	86/196 (43.9)		26/59 (44.1)	0/59	
≥6	86/196 (43.9)		20/59 (33.9)	0/59	0.130
Vomiting	182/196 (92.9)	0/196	55/59 (93.2)	0/59	0.924
Duration (days)		0/182			
1	23 (12.6)		6/55 (10.9)	0/55	
2	59 (32.4)		19/55(34.6)	0/55	
≥3	100 (55.0)		30/55 (54.6)	0/55	0.922
Max per day (n)		0/182			
<5	123 (67.6)		39/55 (70.9)	0/55	
≥5	59 (32.4)		16/55 (29.1)	0/55	0.642
HIV					
Exposed (%)	25/196 (12.8)	0/196	6/59 (10.17)	0/59	0.594
Infected (%)	2/58 (3.5)	138/196*	0/14 (0)	45/59	0.547
RV1					
Vaccinated (2 doses) (%)	194/196 (99.0)	0/196	58/59 (98.3)	0/59	
Unvaccinated (0 dose) (%)	2/196 (1.0)	0/196	1/59 (1.7)	0/59	0.674
Admitted (%)					
Yes	111/196 (56.6)	0/196	37/59 (62.7)	0/59	0.407
Vesikari score (IQR)	14 (12, 16)	3/196	14 (12, 16)	0/59	0.754**
Dehydration (%)					
None	26/196 (13.3)		7/59 (11.9)		
Some (5%)	124/196 (63.3)		34/59 (57.6)		
Severe (10%)	46/196 (23.5)	0/196	18/59 (30.5)	0/59	0.550
IV fluids (%)					
Yes	58/196 (29.6)	0/196	16/59 (27.1)	0/59	0.714
Oral fluids (%)					
Yes	185/196 (94.4)	0/196	57/59 (96.6)	0/59	0.496
Outcome (%)					
Home	194/196 (99.0)		58/59 (98.3)		
Died	2/196 (1.0)	0/196	1/59 (1.7)	0/59	0.677
Anthropometry (mean and SD)					
Whz	-0.59(1.61)	1/196	-0.92(1.65)	0/59	0.181*
WAZ	-0.46 (1.6)	1/196	-0.50(1.28)	0/59	0.877*
HAZ	-0.04 (2.46)	5/196	0.12(2.46)	4/59	0.688*
MUAC	13.48 (1.28)	1/196	13.39(1.12)	0/59	0.628*

SAM	23/194 (11.9)	3/196	6/59 (10.17)	0/59	0.713
Household size		0/196			
≤5	136/196 (69.4)		46/59 (78.0)	0/59	0.201
>5	60/196 (30.6)		13/59 (22.0)		
Previous diarrhoeal admission (%)	15/196 (7.7)	0/196	5/59 (8.47)	0/59	0.829
Previous diarrhoeal presentation (%)	91/196 (46.4)	0/196	36/59 (61.02)	0/59	0.049
Premature (%)	6/196 (3.1)	0/196	2/59 (3.4)	0/59	0.899
Birth weight (mean and SD)	2.96 (0.64)	12/196	2.98(0.51)	3/59	0.872*
Ever Breastfed (%)	195/196 (99.5)	0/196	59/59 (100)	0/59	0.583
Other food (%)	190/196 (97.0)	0/196	58/59 (98.3)	0/59	0.573
Additional child <1					
0 (%)	187/192 (97.4)		59/59 (100.0)		
1 (%)	5/192 (2.6)	4/196	0/59 (0.0)	0/59	0.211
Additional children < 5					
0 (%)	126 (65.0)		40/59 (67.8)		
1 (%)	62 (32.0)		17/59 (28.8)		
2 (%)	5 (2.6)		2/59(3.4)		
4 (%)	1 (0.5)	2/196	0/59 (0)	0/59	0.894
Electricity at home					
Yes (%)	89/196 (45.4)	0/196	28/59 (47.5)	0/59	0.782
Shared toilet					
Yes(%)	148/196 (75.5)	0/196	48/59 (81.4)	0/59	0.351
How long for household to access water (%)					
How long for household to access water (%)					
0-5 mins	34/196 (17.4)		12/58 (20.69)		
5-30mins	94/196 (48.0)		21/58 (36.21)		
>30 mins	68/196 (24.7)	0/196	25/58 (43.10)	1/59	0.286
Water source (%)					
Well	16/195 (8.2)		0/58 (0.0)		
Borehole	35/195 (18.0)		8/58 (13.8)		
Shared tap village/compound	115/195 (59.0)		42/58 (72.4)		
Tap to house	29/195 (15.0)	1/196	8/58 (13.8)	1/59	0.088
How many people have a regular salary (%)					
0	68/195 (34.9)		22/59 (37.30		
≥1	127/195 (65.1)	1/196	37/59 (62.7)	0/59	0.734
Problems getting food in the past month (%)					
No	136/196 (69.4)		38/59 (64.4)		
Sometimes/often	60/196 (30.6)	0/196	21/59 (35.6)	0/59	0.471
Has an adult skipped a meal in the past 2 weeks?					
Yes	45/197 (22.8)	0/197	14/59 (23.7)	0/59	0.902
Wealth (Mean and SD)	2.38 (0.57)	5/196	2.38(0.41)	0/59	0.999*
Time of recruitment					

Quarter of year (%)					
Jan-Mar	43/196 (22.0)		22/59 (37.3)		
Apr-Jun	58/196 (29.6)		18/59 (30.5)		
Jul-Sept	56/196 (28.6)		10/59 (17.0)		
Oct-Dec	39/196 (19.9)	0/196	9/59 (15.3)	0/59	0.069
Season (%)					
In season	114/196 (57.9)		28/59 (47.5)		
Out of season	82/196 (42.0)	0/196	31/59 (52.5)	0/59	0.147
Location (%)					
QECH	113/196 (57.7)		36/59 (61.0)		
HC	83/196 (42.4)	0/196	23/59 (39.0)	0/59	0.646

[†]P values are X^2 p values for differences in proportions between case children and control children unless otherwise specified. *2 sided independent ttest **rank sum test.

Table A5. Comparing those completing the RRTE study to vaccine age eligible rotavirus positive children from the diarrhoeal surveillance study not recruited into the RRTE study

	RRTE study		Diarrhoeal surveillance study		P value
	Summary statistic	Missing data	Summary statistic	Missing data	
Age (Median and IQR)	11.4 (8.7,15.3)	0/196	10.2 (7.6, 14.9)	0/135	0.055**
Sex (male) (%)	108/196(55.1)	0/196	83/135 (61.5)	0/135	0.248
Diarrhoea (%)	196/196 (100)	0/196			
Duration (days)		0/196			
1-3	173/196 (88.3)		107/135(79.3)		
5	12/196 (6.1)		16/135 (11.9)		
≥6	11/196 (5.6)		12/135 (8.9)	0/135	0.078
Max per day (n)		0/196			
1-4	24/196 (12.2)		19/135 (14.1)		
5	86/196 (43.9)		58/135 (43.0)		
≥6	86/196 (43.9)		58/135 (43.0)	0/135	0.888
Vomiting	182/196 (92.9)	0/196	119/135(88.2)	0/135	0.143
Duration (days)		0/182			
1	23 (12.6)		13/119 (10.9)		
2	59 (32.4)		35/119 (29.4)		
≥3	100 (55.0)		71/119 (59.7)	0/119	0.717
Max per day (n)		0/182			
<5	123 (67.6)		86/119 (72.3)		
≥5	59 (32.4)		33/119 (27.7)	0/119	0.388
HIV					
Exposed (%)	25/196 (12.8)	0/196	19/133 (14.3)	2/135	0.689
Infected (%)	2/58 (3.5)	138/196*	4/45 (8.9)	90/135	0.242
RV1 (%)					
Vaccinated (2 doses)	195/196 (99.0)	0/197	123/135(91.1)	0/135	
Vaccinated (1 dose)	0/196 (0.0)	0/197	4/135 (3.0)	0/135	
Unvaccinated (0 dose)	2/196 (1.0)	0/197	8/135 (5.9)	0/135	0.002
Admitted (%)					
Yes	111/196 (56.6)	0/196	110/135(81.5)	0/135	0.000
Disease severity (%)					
(Median & IQR)	14/196 (12,16)	3/196	15 (13,16)	0/135	0.186
Dehydration (%)					
None	26/196 (13.3)		9/135 (6.7)		
Some (5%)	125/196 (63.3)		89/135 (65.9)		
Severe (10%)	46/196 (23.5)	1/196	37/135 (27.4)	0/135	0.144
IV fluids (%)					
Yes	58/196(29.6%)	0/196	45/135 (33.3)	1/135	0.470
Oral fluids (%)					
Yes	185/196 (94.4)	0/196	130/135(96.0)	0/135	0.426
Outcome (%)					
Home	193/195 (99.0)		131/135(97.0)		

Died	2/195 (1.0)	1/196	4/164 (3.0)	0/135	0.195
Anthropometry (Mean and SD)					
WHZ	-0.59(1.61)	1/196	-1.00(1.73)	3/135	0.037*
WAZ	-0.47 (1.6)	3/196	-0.78(1.40)	0/135	0.031*
HAZ	-0.05(2.47)	7/196	-0.133(2.46)	7/135	0.762*
MUAC	13.48 (1.28)	1/196	13.18(1.41)	0/135	0.049*
SAM	23/193 (11.9)	3/196	27/135 (20.0)	0/135	0.045
Household size					
≤5	136/196 (69.4)		102 (75.6)		
>5	60 (30.6)	0/196	33 (24.4)	0/135	0.220
Previous diarrhoeal admission (%)	15/196 (7.7)	0/196	9/135 (6.7)	0/135	0.734
Previous diarrhoeal presentation (%)	91/196 (46.4)	0/196	85/135 (62.9)	0/135	0.003
Premature (%)	6/196 (3.1)	0/196	3/134 (2.2)	1/135	0.652
Birth weight (Mean and SD)	2.98 (0.60)	13/196	2.96(0.52)	11/135	0.808*
Ever Breastfed (%)	195/196 (99.5)	0/196	134/135(99.3)	0/135	0.790
Other food (%)	190/196 (97.0)	0/196	127/135(94.1)	0/135	0.203
Additional child <1 year (%)					
0	187/192 (97.4)		129/134(96.3)		
1	5/192 (2.6)	4/196	5/134 (3.7)	1/135	0.561
Additional children < 5 years (%)					
0	126 (65.0)		114/135(68.9)		
1	62 (32.0)		44/135 (26.7)		
2	5 (2.6)		5/135 (3.7)		
3	0 (0)		1/135 (0.7)		
4	1 (0.5)	2/196	0/135 (0)	0/135	0.493
Electricity at home (%)					
Yes	89/196 (45.4)	0/196	58/135 (43.0)	0/135	0.660
Shared toilet (%)					
Yes	148/196 (75.5)	0/196	104/135(77.0)	0/135	0.749
How long for household to access water (%)					
0-5 mins	34/196 (17.4)		23/134 (17.2)		
5-30mins	94/196 (48.0)		55/134 (41.0)		
>30 mins	68/196 (24.7)	0/196	56/134 (41.8)	1/135	0.385
Water source (%)					
Well	16/195 (8.2)		6/132 (4.6)		
Borehole	35/195 (18.0)		23/132 (17.4)		
Shared tap village/compound	115/195 (59.0)		86/132 (65.2)		
Tap to house	29/195 (15.0)	1/196	17/132 (12.9)	3/135	0.514
How many people have a regular salary (%)					
0	68/195 (34.9)		55/135 (40.7)		
≥1	127/195 (65.1)	1/196	80/135 (59.3)	0/135	0.278
Problems getting food in the past month (%)					
No	136/196 (69.4)		88/135 (65.2)		
Sometimes/often	60/196 (30.6)	0/196	47/135 (34.8)	0/135	0.422

Has an adult skipped a meal in the past 2 weeks?					
Yes	45/196 (23.0)	0/196	99/135 (73.3)	0/135	0.441
Wealth (Mean and SD)	2.38 (0.57)	5/196	2.37(0.50)	2/135	0.918*
Time of recruitment					
Quarter of year (%)					
Jan-Mar	43/196 (22.0)		39/135 (28.9)		
Apr-Jun	58/196 (29.6)		48/135 (35.6)		
Jul-Sept	56/196 (28.6)		29/135 (21.5)		
Oct-Dec	39/196 (19.9)	0/196	19/135 (14.1)	0/135	0.135
Season (%)					
In season	114/196 (58.2)		77/135 (57.0)		
Out of season	82/196 (41.8)	0/196	58/135 (43.0)	0/135	0.838

[†]P values are X^2 p values for differences in proportions between case children and control children unless otherwise specified. *2 sided independent ttest **rank sum test.

Table A6. Comparison of Anthropometric measurements from the RotaRITE transmission epidemiology study, and values from the 2010 Blantyre Demographic and Health (DHS) Survey

	RRTE Values			Blantyre (DHS 2010)			Standard deviation	
	Mean value	%<-2sd	%< -3 sd	Mean value	%<-2sd	%< -3 sd	RRTE	WHO*
HAZ	-0.04	19.4	9.42	-1.6	41.6	20.5	2.46	1.35-1.95
WHZ	-0.59	17.9	5.10	0.4	2.2	0.0	1.61	1.08-1.50
WAZ	-0.46	10.2	2.04	-0.7	12.7	2.5	1.61	1.17-1.46

Where HAZ represents height for age Z score, WHZ weight for height Z score, and WAZ weight for age Z score. *Range for standard deviation given as acceptable by WHO(355)

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